

Learning to live in a greenhouse

The dictum about not throwing stones in greenhouses may be generally applicable in the years ahead. Incoherent bad temper is on the increase, while even well-intentioned official committees may make things worse.

THE property correspondent of the British newspaper *The Financial Times* was quick off the mark last Saturday. "Amid all the talk of the greenhouse effect", he wrote, "there is a particular problem for estate agents [otherwise 'realtors'] in low-lying parts of the UK" — that of "allaying fears . . . of inundation" among potential house and land purchasers. Noting that much of eastern England is either at or below sea level, he went on to declare that "the risk, in fact, is minimal . . . the Dutch long ago showed that modern engineering with sufficient funding is up to the task". It will be interesting to see what they make of that in Bangladesh.

Meanwhile, in Britain and the rest of Europe, the heat seems to be getting to people. Prime Minister Margaret Thatcher's promise that Britain's greenhouse emissions will be no greater in 2005 than in 1990 has been denounced by environmental groups as inadequate. The European Communities have, rather, the same objective for the end of this decade, largely on the strength of a conference in the Netherlands last year that the British government ostentatiously chose not to attend. But there is no formal agreement yet, no mechanism by which European compliance might be achieved and, importantly, no set of yardsticks by which compliance and/or failure to comply might be assessed, let alone sure knowledge of how governments might best comply.

If the excess greenhouse effect proves to be real, that will be bad enough, but the bad temper the prospect seems certain to engender will be something else unwanted. No purpose will be served by a competition between national governments to be first with self-denying regulations on carbon dioxide and other gases — the result would simply be to reduce the pressure on others. The need is for the framework of a convention. Without that, self-denial is like unilateral disarmament.

Nor does it help that well-intentioned bodies such as the Response Strategies Working Group of the UN Intergovernmental Panel on Climate Change put out such woolly statements as its first draft summary report (prudently labelled "Do not quote"). The group correctly concludes that a convention is the central need, and similarly recognizes that the disparity between rich and poor countries is inseparable from the greenhouse problem. But then, perversely, it argues that the intellectual property rights of companies from developed countries should be disposed of as the recipients of technology think

fit, apparently ignorant of the importance of just that issue in the UN-sponsored negotiations now under way in the General Agreement on Tariffs and Trade (GATT). It is to be hoped that the working group will have done better by November. □

Who makes mistakes?

The mistakes that researchers make are dwarfed by the mistakes of other professionals, for which we pay.

MISTAKES will happen, but some have greater consequences than others. That is worth bearing in mind when the research profession has often been criticized for the appearance of erroneous research reports in the literature. That is why researchers and their critics may be respectively heartened and chastened by some of the spectacular mistakes that have recently occurred in other professions, notably in accountancy and the law. The difference is that when research reports are in error, it is science that suffers; when accountants or lawyers make mistakes, the costs are met by the rest of us.

Last week, the British banking system put an end to a company called B&C (for 'British and Commonwealth'), one of the stockmarket successes of the 1980s, which had made the mistake of paying, 18 months ago, roughly £500 million for a company called Atlantic, whose business of leasing computers had apparently been sustained by its practice of defining future obligations as current profits. When the truth began to trickle out, two months ago, B&C felt obliged to 'recapitalize' Atlantic to the tune of what it had paid for ownership in the first place, which turned out to be not very different from the net worth of the collection of secondary banks and financial services companies which were the mainstay of its business. It is not surprising that B&C's creditors decided last week that the company was not merely skating on thin ice, but on liquid water, and pulled the plug. One irony, for those who hold that the future lies in the creative marriage of accountancy and technology, is that B&C happened by accident to have acquired a half share in the biotechnology company Celltech, at what was believed in 1980 to be the cutting edge of high technology, but which is now embarrassed by the prospect of a firesale of its shares.

The case (again British) of the electronics company



Ferranti, although smaller, is on the face of things more serious. Ferranti bought a US defence electronics company in 1988 and alleged last summer that it had been induced to pay \$200 million above the odds because supposed defence contracts with third parties were figments of creative imagination. Ferranti, which is technically an excellent enterprise, nearly went under and is now much emasculated by enforced disposals. In each case, non-technical professionals, mostly accountants, appear to have failed to recognize a house of cards when asked to look at one. In both cases, strategically important technical enterprises seem to have been damaged by non-technical negligence. Apart from those employed, the chief losers are the 'shareholders' who nowadays are present or would-be pensioners and insurance beneficiaries.

There is no great lesson to be drawn from these affairs — only a little irony. In science, some pride may derive from knowing that more exacting standards of prudence apply to research than to other professional activities, but should the difference be so great? Imprudence on this scale is not exclusively British — the \$500,000 million US taxpayers will have to spend in the next decade making up for imprudent arrangements of domestic mortgages would be enough to breathe new life into the Soviet Union, Eastern Europe or perhaps both. □

Extreme prejudice

Europe and the United States have been too quick with scepticism of the latest offer of collaboration from Japan.

THE Japanese scheme for an international effort to develop intelligent manufacturing systems (IMS) has run into the troubles (see page 563) that plagued the Human Frontiers Science Programme (now rescued from scepticism, but with less to spend). First, there is incomprehension; people elsewhere say they do not understand what is proposed. Then there is suspicion; people elsewhere say that Japan must be plotting yet another way of reinforcing its industrial supremacy. Finally, there is a reaction — heavyhanded determination by the United States and the European Communities that the project should not go ahead in the form proposed, or on terms substantially different from the collaborative projects with which they are themselves familiar. The result may be to turn an imaginative project into dust and ashes.

That people do not readily understand Japan is a forgivable commonplace, but in their own interests they should try harder. By occidental standards, the original version of IMS was vague: "let there be an international collaboration to develop new manufacturing technology". The original idea came from the University of Tokyo, with support (which meant publicity, not money) from the Ministry of International Trade and Industry (MITI). What can such a vague description mean? The headphones attached to cassette players now attached to many young people's heads may have sprung from some

research director's demand "let us make a portable music system". (The word "Walkman" would have come later.) Envious Western manufacturers would profit from asking themselves some Japanese-style general questions.

The paranoia with which the Japanese proposal has been greeted is similarly misplaced. IMS was meant to be a club to which manufacturing companies would individually subscribe. There would be some MITI money to oil the wheels, perhaps even an international institute. But the US government has taken offence that US manufacturers have been approached directly, not through diplomatic channels, while the European Commission fears that companies from member states would be fleeced of their intellectual property with the first yen of MITI money that came their way. So IMS has been turned into a government-supervised project hardly different from European Eureka.

The plain truth is that the non-Japanese companies likely to be interested in the venture are well able to look after themselves, but stand to gain from collaboration of this kind of access to markets dominated by the Japanese. In its original form, IMS also offered ways in which technologists from elsewhere might learn at first hand how Japanese companies set about research and development, thus turning the US government's standard complaint that Japan is too secretive in this regard. To have responded with such suspicion to the proposal can only confirm Japanese industry in the belief that it has nothing to fear from its nominal competitors elsewhere. □

Pre-publicity

Journals like this frown on pre-publicity for what they publish, but there must be exceptions.

THE development of a potential subunit vaccine for AIDS, reported on page 622, is clearly important. But can it have been proper that Genentech should have made the gist of this discovery available to its shareholders two weeks ago, in advance of a shareholders' meeting to decide whether the proposed merger with Roche should go ahead?

The circumstances are these. The authors of the research enquired three weeks ago whether they would be able to use the information as they did, saying that they were aware of the policy of this journal (and others) that prior notice of publication should not be formally provided and offering to withdraw the research report if shareholders could not otherwise be informed. This journal's restrictive policy on prior publicity derives from a wish not to be pre-empted by less substantial sources of information — in *Nature* one day but in the *New York Times* the day before, for example. But the policy is not rigid, and may be broken when there is information of urgent public importance to report. Moreover, the policy is not intended to (and should not) inhibit discussion at regular scientific meetings. Do not shareholders also have rights? □

Utah faculty protest cold fusion dealings

- Cold fusion institute funding probed
- Fusion critics receive legal threats

Washington

FINANCIAL and legal activities at the University of Utah, involving a \$500,000 donation to the National Cold Fusion Institute (NCFI) and payments to a lawyer who sent letters to some faculty members threatening legal action on behalf of Stanley Pons and Martin Fleischmann, have brought protests even from supporters of cold fusion research. Reviews are to be made of scientific and financial activities of NCFI, which was set up last year on the University of Utah campus with \$5 million from the state of Utah.

On 29 March this year, during an NCFI conference held a year after the first cold fusion press conference, *Nature* published a paper in which Michael Salamon, a University of Utah physicist, and nine co-authors described the entirely negative results of a five-week search for nuclear evidence of fusion reactions coming from electrolytic cells in Pons' own laboratory. Five days later, on 3 April, Salamon received a letter from C. Gary Triggs, an attorney in Morganton, North Carolina, acting for Pons and Fleischmann, which declared that "the paper as published is untenable" and "should be voluntarily retracted." In his letter, which was also sent to eight of Salamon's nine co-authors, Triggs said that he had been "instructed by my clients to take whatever action is deemed appropriate to protect their legal interest and reputations".

Nature itself received a long letter from Triggs a year ago, soon after Pons and Fleischmann had been told that their initial paper presenting evidence for cold fusion was not acceptable for publication without further revision. Triggs' letter listed a number of reasons why Pons and Fleischmann were unhappy with *Nature's* decision and other actions, but after a correspondence between Triggs and *Nature's* lawyers the matter was dropped.

In his letter of 3 April to Salamon, Triggs also refers to "actions of *Nature* to sensationalize the negative results and publish the same immediately prior to the beginning of the recent conference." Triggs has, however, not written to *Nature* to say what actions he is referring to. Salamon's paper appeared during the cold fusion conference quite by coincidence, having been accepted for publication at the end of January, before *Nature* was aware of the conference.

Salamon says he undertook the investigations in Pons' laboratory at the request of the university, but that after receiving

Triggs' letter he was told that legal representation by the university "could not be guaranteed". Later he discovered that, as a state employee, he is automatically indemnified against legal costs or actions arising from official business. After consulting privately with a lawyer, he decided not to reply to Triggs' letter.

After investigations by Gary Taubes, author of *Nobel Dreams* and of a forthcoming book on cold fusion, Tim Fitzpatrick, a reporter for the *Salt Lake City Tribune*, revealed that Triggs had been paid \$68,000 by the University of Utah for work related to cold fusion patents. At this revelation, says Salamon, university faculty members "got incensed."

Their ire soon increased. Taubes and Fitzpatrick also discovered that a \$500,000 gift to NCFI, advertised to prospective investors as an anonymous donation, had in fact come from the University of Utah Research Foundation through the office of University president Chase N. Peterson. Twenty-two faculty members then signed a letter asking for a scientific

Disappearing tritium

At Texas A&M University last week, the evidence for cold fusion suffered a sudden loss. Kevin Wolf announced that his claims that some electrolytic cells had generated tritium must be withdrawn because tritium contamination had been discovered in the palladium he was using. Palladium from the same supplier was also used in some experiments done by a second Texas A&M team, led by John Bockris, and one of several claims of tritium production in experiments at Los Alamos National Laboratory (LANL) turned out to have involved palladium from Texas A&M.

Without some indication that tritium, helium, neutrons or gamma-rays are also produced, physicists have generally ascribed still-extant claims of heat production to poor measurement. Wolf, and Ed Storms and Carol Talcott at LANL, detected tritium in several experiments, and although they made no claims for heat production their results were widely cited as evidence for 'cold' nuclear processes. Bockris and his group say they have produced both heat and tritium.

Neither Bockris nor Storms and Talcott are ready to abandon all their claims for tritium production. Both groups used palladium from a variety of sources, and in the LANL experiments palladium samples were heated to drive off any absorbed tritium before experiments were done.

D.L.

review and financial audit of NCFI, and questioning whether Peterson's role in the mislabelling of the \$500,000 might warrant his resignation. Salamon also says that, due especially to the efforts of Joseph Taylor, vice-president for academic affairs, the university has now guaranteed him legal indemnification.

On 5 June, Salamon received a second letter from Triggs, apologizing for "any concerns or misconceptions" his first letter may have caused and assuring him that there was no intent "to limit in any way the lawful exercise of your academic freedom." The letter adds that Pons and Fleischmann now "intend to settle [the] dispute in the court of science through publication." But the letter repeats Triggs' earlier complaint that the publication of Salamon's paper on the opening day of the cold fusion meeting "certainly seemed" to be "another in a long series of negative acts by *Nature* to damage cold fusion in general and my clients in particular".

James Brophy, vice-president for research at the University of Utah, says that invoices from Triggs to the university show that the lawyer was paid only for patent-related work. He explains that although Triggs is not a patent lawyer, he received approval to work with Pons and Fleischmann on patent applications.

Brophy says also that Salamon "got the wrong impression" when he asked if the university would indemnify him. "The question was not asked in that way," says Brophy, explaining that because there was no lawsuit, only a letter threatening legal action, when Salamon approached the university administration, the question of indemnification "was moot".

Confusion over the origin of NCFI's "anonymous" \$500,000 gift is also understandable, says Brophy. The University of Utah Research Foundation, whose income derives in part from patent licenses and royalties, routinely makes money available to the university president's office, to be used at Peterson's discretion. In this case, says Brophy, Peterson gave the money to NCFI director Fritz Will without saying where it came from. Peterson has said there was "no intent to deceive" but admits that "in retrospect it was a mistake" not to make the money's provenance clear. On 11 June Peterson, referring to the request made to the governing boards to examine his presidency, announced that he would retire at the end of the coming academic year.

NCFI will now face two separate reviews. The university's state steering committee is to set up another committee to select panel members who will review NCFI's scientific work. Another committee will be set up to perform a fiscal audit but this, Brophy says, would have happened anyway because NCFI undergoes financial audit yearly. **David Lindley**

Hot air — or what?

Boston

STEPHEN Schneider, of the National Center for Atmospheric Research, faced off against Richard Lindzen of Massachusetts Institute of Technology (MIT) last week in a debate at MIT entitled "Is it getting hotter or what?" Schneider supports the view that current climate models can reasonably predict a mean rise in global temperatures of 2 °C or more over the next century; Lindzen is an increasingly vociferous critic of the inadequacies of current climate models.

At the debate, Lindzen argued that current models are weak, and that a warming trend remains to be seen in the recent temperature data. The picture presented by Schneider and others is "misleading," principally because present climatological models are "inconsistent with observations of landbased global average temperatures", contain documented errors, and have made a poor job of predicting climate change in the tropics.

Furthermore, Lindzen said, climate models do not adequately account for the role of feedback from the oceans. Lindzen stressed the role of convection in the upper atmosphere that allows heat to escape by circulating vertically. Those who predict global warming, he maintained, emphasize only the atmosphere's radiative features for heating and cooling the Earth. "The effectiveness of our greenhouse is just 25 per cent", he said, adding that if it were much more effective, the Earth's temperatures would be far higher — in the vicinity of 77 °C. In particular, Lindzen cited research on the transport of water vapour in the atmosphere, stating that current transport models are "off by a factor of two." Lindzen maintains that the parameters used by current models "exaggerate the moisturization of the upper troposphere". In the models, he says, much of the predicted warming comes from atmospheric humidity which traps the Earth's heat. Given the extent of the many uncertainties, Lindzen stated, cause for alarm should come only when observed temperatures are shown to clearly be higher than can be predicted by normal deviation.

In his rebuttal, Schneider acknowledged that the issues raised by Lindzen are legitimate, and questioned whether the combined effects of different errors could all be working together, but might instead "cancel each other out." Despite all the uncertainties presented, Schneider felt that it is "unlikely that the models are off by more than a factor of three," a fact which would greatly alter the perceived increase in temperature, but not negate altogether the possibility of a significant temperature increase.

But on balance, even these two re-

searchers, representing the far ends of the spectrum of current debate within the community of atmospheric researchers, ended up agreeing on more points than they might have otherwise acknowledged. Schneider readily acknowledged that current models are neither particularly accurate nor reliable. Lindzen, for his part, agreed upon the need for intensified research efforts, if only "to find out why the models are wrong". As for the policy questions of how to respond in the

IPCC GLOBAL WARMING REPORT

Urgent action still opposed

London

WORKING group III of the Intergovernmental Panel on Climate Change (IPCC) emerged at the end of its Geneva meeting last week with its suggestions for carbon dioxide emissions fundamentally unchanged from the widely-criticized measures outlined in an early leaked draft of the report (see *Nature* 345, 373, 31 May 1990). The US-chaired group III examined possible responses to the threat of global warming and has been accused by environmentalists of ignoring scientific evidence and simply following the policy of member governments.

In response to pressure from governments already committed to emission cuts and the consensus arrived at by the climatologists of working group I, the repeated references in the leaked draft to the "scientific uncertainties" surrounding global warming are reduced in the final version. But Paul Hohnen from Greenpeace International, an observer at the meeting, says group III's report may still be "all things to all persons", and provide

CFCs

Britain going CFC-free?

London

BRITAIN could eliminate almost all chlorofluorocarbon (CFC) use by 1997 at "relatively little cost", according to a report sponsored by the Department of Trade and Industry. A team of consultants led by Coopers & Lybrand Deloitte found that British CFC use had already halved between 1986 and 1989.

Although use of ozone-damaging halons had increased slightly over the same period, trade and industry minister Eric Forth said Britain had met its commitment to the Montreal Protocol "almost ten years ahead of requirements". The report would be made available to delegates attending a meeting in London later this month to strengthen the Montreal Protocol, he said.

Peter Aldhous

face of such uncertainty — these were left for several social scientists who spoke later in the event's programme. Amidst sketchy discussion of the possibility of such things as a "world carbon budget", perhaps the best prescription came from MIT economist Henry Jacoby. If the threat of global warming can wean the planet off of its harmful greenhouse gas-producing ways, he said, it can thereby encourage us to do those things we probably "ought to be doing anyway." As Jacoby put it, in the face of potential global warming the answer is evident: "repent."

Seth Shulman

an excuse for inactivity on the part of governments reluctant to curb emissions.

The Geneva meeting again saw US, Canadian, Soviet and Japanese representatives (joined by oil-rich Saudia Arabia, in its first significant contribution to the global warming debate) opposing Western European countries that want to set reduced greenhouse gas emissions targets early on. Scandinavian nations, backed by others including the Netherlands, France and Italy, tried unsuccessfully to insert into the report a line noting that some countries want negotiations towards a protocol on greenhouse gases to start immediately — rather than waiting for IPCC to draw together the conclusions from its three working groups.

European Communities (EC) environment ministers, meeting last week in Luxembourg, delayed setting targets on greenhouse emissions. Together with environment commissioner Carlo Ripa di Meana, West Germany, France and Holland had called for EC member states to stabilize emissions at current levels by 2000. But Britain, Spain, Portugal and Greece refused to back this measure, ministers instead adopting an Irish suggestion for countries to establish national strategies to curb emissions, before discussing the issue again in October.

Before the EC meeting, British Labour opposition environment spokesman Bryan Gould labelled prime minister Margaret Thatcher's recent pledge to stabilize UK emissions at present levels by 2005 as "a gigantic confidence trick". Thatcher's statement that this target involved undercutting projected emissions by up to 30 per cent was based on a "far-fetched" Department of Energy study, which assumes that Britain will recover all of the energy-intensive heavy industry lost during the economic recession of the 1970s, Gould said. He added that a Labour government would stabilize emissions by 2000, but did not say exactly how this would be achieved.

Peter Aldhous

Gift horse arouses suspicion

Tokyo

WHEN a couple of Japanese academics backed by Japanese industry and the Ministry of International Trade and Industry (MITI) launched the Intelligent Manufacturing System (IMS) project earlier this year, they thought it would help ease trade friction and offer a way for Japan to pay back its technological 'debt' to the Western world.

But government officials in Washington and Brussels have taken an altogether different view. They see the project as yet another attempt by Japan to take over the world and have forced major changes.

IMS is the brainchild of a committee of academics, industrialists and MITI officials headed by Hiroyuki Yoshikawa, dean of the faculty of engineering at Tokyo University. The committee proposed an international research centre in the United States or Europe with funding over ten years of about \$1,000 million. Japanese private industry and government was to provide about 60 per cent of the total and other countries the rest.

The institute, with researchers drawn from academic institutions and industry around the world, was to have as its aim the development of new automated, standardized manufacturing systems covering all stages of manufacturing from design to retail and distribution. As Japan is a world leader in manufacturing technology, the committee (and Japanese industry) felt that Japan had much to offer. Several large US companies and the US-based Society of Manufacturing Engineers registered their approval.

But officials of the US Department of Commerce (DOC) and the European Communities (EC), see IMS as an attempt to tap Western expertise in systems integration and software. Commerce department officials are also piqued because Japan ignored government and went straight to US industry and the Society of Manufacturing Engineers for support. And the EC Commission has actively prevented European companies from joining the project, a MITI official claims.

Two major US companies, Rockwell International Overseas Corporation and United Technologies Pratt and Whitney, and the Japanese subsidiary of IBM, IBM Japan, have signed up for the project along with 62 Japanese companies covering almost all sectors of manufacturing, including FANUC (one of the world's leading manufacturers of machine tools and robots), Toyota, Nissan, NEC, Hitachi, Toshiba, Fujitsu, Sharp, NKK (a major steel producer), several companies from the giant Mitsubishi group and Kawasaki Heavy Industries.

These 'core' companies have donated ¥12 million (\$80,000) each to finance the

conceptual design of IMS. An IMS office was established in Tokyo earlier this year at a cost of over ¥100 million. And in April the office began advertising for research proposals from private companies and public research institutes around the world (*Nature*, Classified, 19 April).

But a few days before the advertisements appeared, the EC Commission announced a counterproposal called the Future Generation Manufacturing Systems (FGMS) project. Also in April, DOC hosted a contentious meeting with 50 US companies to discuss the project and an assistant secretary of commerce criticized the programme at a congressional hearing, describing it as an attempt by Japan to target US industries.

A meeting between US, Japanese and EC officials was held last month in Brussels. Japanese officials agreed to adopt many of the conditions of the EC's FGMS proposal and also gave DOC an equal say in the project. The result was the cancellation of the meeting, planned to take place in Tokyo last week, at which an international

SUPERCONDUCTING SUPER COLLIDER

US wish-list secrets

Washington

FOREIGN participation in the \$8,000 million Superconducting Super Collider (SSC), if it were to come in the form of a large cheque, would be immediately welcomed by the US Congress. But the idea that foreign governments or industries might instead prefer to do some of the high-technology manufacturing that was supposed to be a benefit of the SSC to US industry has caused political unease. Nevertheless, the team from the US Department of Energy (DOE) that visited Japan earlier this month presented Japanese government officials with a list of possible areas of participation, including construction of the superconducting main-ring magnets (see *Nature* 345, 467; June 1990). A Japanese version of the list, which the DOE team told the press was secret, has been obtained from government officials and passed to *Nature*.

Devices Japan can provide for SSC: (1) Half of SSC's superconducting magnets (including collider ring magnets and dipole and quadrupole magnets for HEB*).

(2) Components for large SSC particle detectors

(3) Other possible fields (a) Collider and HEB [High Energy Booster, the penultimate accelerating ring, which feeds protons into the main ring.] ultra-low temperature equipment (half); (b) Power supply components for magnets; (c) Ultra-low temperature connectors (spool parts); (d) Iron and steel parts; (e) Magnets

committee of academics was to examine research proposals (see *Nature* 343, 496; 1990) and map out details of the project. Instead, the United States and EC are to deal separately with projects from their own participants.

Before the change was announced, the IMS office had received nearly a hundred proposals, 68 from Japan, 14 from North America, 12 from Europe and 1 from Australia. Most of the proposals (56) come from private industry, the rest (39) from public research institutes.

The United States, EC and Japan will each put forward projects at a September meeting in Tokyo. According to the Brussels agreement, each collaboration "must provide a balanced flow of knowledge". The revisions have converted what was intended by Japan to be largely a private-sector initiative into a highly political government-controlled project. MITI officials do not seem to be concerned about this change, but Japan's private industry, which required considerable persuasion to begin the project in the first place, is beginning to have second thoughts about contributing to the project.

David Swinbanks

for large detectors; (f) Support construction of underground tunnel. And provide boring machines; (g) Money.

The items on the list (note that one of them is not strictly speaking a 'device') were assembled by DOE officials with the assistance of SSC director Roy Schwitters.



An SSC spokesman said that US manufacturers with whom SSC physicists and engineers have been in contact had taken a "statesmanlike attitude" to the idea that other countries might help build some parts of the 20 TeV accelerator, and that worries over technology transfer were largely a Washington problem.

David Swinbanks & David Lindley

Soviets enact new law

Moscow

THE Supreme Soviet of the USSR has now approved a new law on AIDS and a procedure for implementing it with effect from the beginning of next year. It also intends to set up a special government commission, to be headed by a vice-chairman of the Council of Ministers, to work out by then a national programme to combat AIDS.

Although the first AIDS case was registered in 1987, the order then issued by the Supreme Soviet for a national prevention programme was never implemented, according to Vladimir Pokrovsky, president of the Soviet Academy of Medical Sciences.

Even so, AIDS is not much of a problem in the Soviet Union. Since 1987, 55 million people have been checked for HIV (human immunodeficiency virus) infection, according to Igor Denisov, the newly appointed Health Minister. (He replaces Academician Yevgeny Chazov, who has resigned.) HIV infection was found in only 984 people, of whom 502 were foreign nationals. There have been 29 AIDS patients, of whom 19 have died.

Concern nevertheless persists because Soviet health-care establishments are poorly equipped and their personnel ill-qualified (as well as being capable of showing criminal irresponsibility and negligence). It remains horrifying that children have been infected with HIV in clinics in Elisla, Rostov-on-Don, Volgograd and Stavropol (with mothers acquiring infection from their babies). Oddly, 57 per cent of those infected with HIV are children.

The shortage of single-use syringes remains acute, with their importation limited by lack of foreign currency. The factories meant to make them under foreign licence are still not in operation. There is also an urgent need of azidothymidin (AZT), which is produced in limited quantities for lack of raw materials. The Supreme Soviet was told that a plan to produce AZT from salmon soft roe (which is plentiful at fish canneries across the country) had been considered by the Council of Ministers last year. Boris Vinogradov, a researcher from Kamchatka in the Soviet Far East, has produced a biologically active preparation from marine products. The USSR Health Ministry and the Biotechnologia Research and Production Group have shown interest in the work, but more support is needed.

Although the Soviet public has been repeatedly told that prostitution, drug addiction and homosexuality did not exist, they have in reality flourished, providing the conditions for a rapid spread of HIV. Expert forecasts, disclosed to the Supreme Soviet by Denisov, predict 1,625

cases of HIV infection by the end of this year, 24,000 by 1992 and, unless the trend is reversed, more than a million cases by 2000.

Against that sombre background, there are doubts that the new law will be sufficient, even though it differs in important ways from the 1987 ordinance. Among other things, the law will make the neglect of professional duties by medical and pharmaceutical personnel a criminal offence, as will be the disclosure of medical secrets. (Such disclosures have led to HIV carriers being ostracized and persecuted.)

But the new law will offer no guarantee of complete safety to patients at Soviet clinics and outpatient clinics. Instead, those infected with HIV at medical establishments are offered a pension before reaching retirement age, which may be poor consolation for a person undergoing treatment for, say, a duodenal disorder who acquires HIV infection in the process. It is hardly surprising, as the Supreme Soviet was told, that the press is warning readers of the dangers of the Russian obsession with seeing doctors and of asking for medication. The new law classifies infection of medical and pharmaceutical personnel with HIV as an occupational disease; such people will be entitled to various allowances and benefits.

The law also provides for the protection of the rights of HIV carriers and AIDS sufferers. They cannot be dismissed from or refused employment or admission to medical and educational establishments, nor can children be refused admission to pre-school establishments if they are HIV carriers or have AIDS. The law also prohibits other infringements of the rights and legitimate interests of HIV carriers and of their next of kin, but specifies certain benefits, such as free transportation to a place of treatment and free medicines for outpatient treatment.

But the law also demands responsible behaviour from them. Once a person knows that he (or she) is an HIV carrier or AIDS sufferer, he can be legally prosecuted for deliberately putting other persons at risk of infection.

Although the new law was approved in a now-rare display of unanimity by the Supreme Soviet, it was much debated. Thus people's deputy Yuri Shcherbak, an epidemiologist with 30 years professional experience, strongly attacked the provision in the draft law providing for obligatory check-ups of Soviet citizens and foreign nationals living in the Soviet Union.

Shcherbak's case was subtle. The draft law required that people should undergo medical checks on the decision of health-care authorities if there were sufficient reasons to assume that they might be HIV carriers, and could be delivered to the

health care establishments by order of a public prosecutor. But who will determine the sufficiency of such reasons, Shcherbak asked? Are there such reasons except the HIV test itself? This seems to be a vicious circle which can be broken only by the use of HIV tests as a preventive measure for every person at a clinic. Otherwise, that provision may lead to malpractice and turn into a kind of "punitive medicine" (as was the case with Soviet psychiatry not so long ago).

On the treatment of foreign nationals, the new law says that those who avoid checks can be deported from the Soviet Union on the decision of health-care authorities. Diplomats can be requested to undergo HIV tests only with their consent, which must be requested in advance by the Soviet Health Ministry with the Foreign Ministry.

The question also arose during the debate of the Chernobyl accident; fears were expressed that there may be a link between HIV and abnormally high exposure to radiation. Previously, it had even been suggested (but flatly rejected by most experts) that the very appearance of AIDS might be linked with enhanced radiation. Pokrovsky does not share this view, believing it to be purely theoretical.

The new law on AIDS is badly needed, but cannot by itself be a radical solution to the problem. This is why the USSR Supreme Soviet instructed the Council of Ministers to ensure the participation of Soviet research institutions in the international effort to deal with AIDS, primarily the effort to find effective methods of treatment.

**Yuri Kanin
Novosti**

STANFORD UNIVERSITY

Gorbachev thanks Panofsky

Washington

SPECIAL thanks were given by Soviet President Mikhail Gorbachev to Wolfgang Panofsky, director emeritus of the Stanford Linear Accelerator Center (SLAC), Sidney Drell, deputy director of SLAC, and William Perry, professor of engineering systems, during his visit to California on 4 June. All three scientists have been associated with Stanford University's Center for International Security and Arms Control; Panofsky as a founding member, Drell as a former co-director and Perry as a co-director. Gorbachev thanked them for working with "patience and perseverance" on the "basic principles of such concepts as international security and strategic stability". His remarks came during an address at Stanford University, introduced by its president Donald Kennedy as the university with the "country's longest accelerator and its best women basketball players".

Alun Anderson

SOVIET SCIENCE

Charting new territory

Santa Clara, California

A 15-member delegation of Soviet science officials met members of the US high-technology industry in Silicon Valley last week as part of the first Soviet Silicon Summit, a conference held to establish science agreements between the two countries.

For US participants, the message from the Soviet delegation was simple: Soviet science is better than its reputation suggests but Soviet scientists need help to commercialize their inventions.

Unfortunately, Soviet scientists do not have the hard currency to pay for the help they need. They are, however, willing to sign barter agreements. "We are great people, but we're out of luck and we have to correct our mistakes", said Nicolai Kleshev, director of the International Center for Informatics and Electronics.

As part of planned Soviet free-market reforms, 2,000–3,000 technical cooperatives, or small laboratories, will become part of the private sector in July. That means that government subsidies are likely to be reduced or abandoned and the centres will have to earn their own keep, either by licensing their technology or by using it to create products. To succeed, the laboratories need Western technology, especially computers and semiconductor production tools. But without hard cash, they can offer only basic research, collaborations and commodities that are not in short supply.

For US companies, the idea of barter is new. Goodwill towards the Soviet Union is in good supply, but concern for the bottom line makes 'non-traditional payment' difficult. Adept Technology, a Silicon Valley robotics company, recently turned down an offer to trade 500 robots for a shipment of caviar and vodka.

Nevertheless, by the end of the summit, Soviet scientists had claimed some trading successes, albeit in more traditional veins.

A unique deal was signed with Cypress Semiconductor Corporation to manufacture Soviet-designed high-definition television and signal-processing chips in the United States. Cypress will pay fees to the Soviet Union on sales of the chips, which the company says could amount to \$60 million per year.

Other agreements are expected. Although the commercial microelectronics industry in the Soviet Union is far behind that in the West, some basic semiconductor research supported by military funds has kept pace. Soviet laboratories had 1-megabit DRAM chips in limited production before US companies first produced their initial runs of the chips in 1986. Soviet scientists claim to be ahead in certain cyclotron techniques for the fabrication of submicrometre semiconductors, some laser technologies, X-ray optics, ion

sources and laser interferometry. "Patent searches were done and no similar developments were found in the West", says Kamil Valiev of the Institute of Physics and Technology.

Almost all the technologies are for sale but US buyers are cautious. "You have to feel a little sceptical", says Nicholas Gralenski, staff scientist for Watkins-Johnson, a California high-technology company. "If the Soviet economy is so weak, how can their technology be strong?" Like many other participants at the conference, he admits that he is more interested in the Soviet Union as a market than as a research partner.

There are between 600,000 and 800,000 IBM-compatible personal computers in the Soviet Union, well behind the 1.1 million that the Soviet five-year plan says should be installed by now.

Atari Corporation is negotiating an arrangement to ship home computers to the Soviet Union in exchange for Soviet-made 256K memory chips. And Soviet officials at last week's meeting suggested that US companies could capitalize on the Soviet Union's traditional strengths in mathematical and theoretical sciences. When supplied with modern computers, Soviet scientists have produced impressive software. Soviet officials ask: why not ship computers to Soviet laboratories and get software back in return?

Although the Soviet scientists at the

summit were new to the art of the deal, they made it clear they would avoid earlier mistakes. Bausch and Lomb made a fortune from a soft contact-lens polymer that came from the Czechoslovakian Academy of Science in the 1960s, but the Czech government took most of the one-time license fee and the original laboratory received little in return. When Bausch and Lomb recently licensed a natural collagen corneal shield from the Moscow Institute for Eye Microsurgery, it found a new awareness of market savvy. The shield agreement sets royalties based on sales, rather than a single fee, and a sizeable percentage of the money will return to the institute.

As free-market reforms pass financial responsibility to individual laboratories, more will demand full proceeds from their patents, says Gordon Feller, editor of the *East-West Report*, a US-Soviet trade newsletter. "There is still a foginess about data ownership", he says, "Who owns the patent? The inventor, the laboratory, or the government?" Ambiguity on that issue has led at least one Soviet laboratory to ask a Western company to sign a confidentiality agreement on an agreement so the laboratory would not have to share the proceeds with its ministry. "The legal structure and framework in the Soviet Union is changing as we speak," said Jeffrey Armstrong of Global Development Corporation, which sponsored the conference. "The rules are written by virtue of the deals that get done."

G. Christopher Anderson

HUMAN EMBRYO BILL

Still some life in the 'pro-lifers'

London

As the Human Fertilisation and Embryology Bill nears the end of its stormy passage through parliament, the UK government is being accused by Dame Mary Donaldson, chairman of the Interim Licensing Authority (ILA) which currently oversees embryo research, of giving "undue weight" to the views of a minority of 'pro-life' Members of Parliament (MPs), in its plans to set up a statutory authority to replace the ILA. Donaldson fears that the new authority will lack the experience necessary to make informed judgements on whether to grant licences for research.

The ILA was set up by the Medical Research Council and the Royal College of Obstetricians and Gynaecologists in the wake of the 1984 Warnock Report on human embryology (on which the bill is broadly based), to provide a voluntary framework of licensing for scientists involved in embryo research, and clinics offering *in vitro* fertilization (IVF) services.

Donaldson attacked the government's plan to set up a separate "embryonic" statutory authority to work alongside the ILA. The statutory authority will take over

full responsibility for licensing embryo research and IVF by the summer of 1991. For the sake of continuity and to use ILA's experience, she said it would be better for the ILA to evolve into a statutory body by the gradual replacement of its members with government appointees.

The ILA has a majority of members from a scientific or medical background. MPs opposed to embryo research say the ILA is biased in favour of research, and do not want it to be given statutory powers for licensing under the bill. For the sake of a "quiet life", Donaldson said, the government had given in to this minority of MPs.

But one MP, who sat on the House of Commons committee which has debated the bill and voted in favour of continued embryo research in the recent Commons vote, says that there is "a strong lobby of opinion" among MPs, backing the government's plans to set up a separate authority. The bill states that the new authority must have a lay chairman and deputy chairman, and less than half of its members must be medical practitioners or have been involved with embryo research or IVF.

Peter Aldhous

BSE: UNITED KINGDOM

Ministerial madness avoided

London

A TRADE war that would have made a mockery of the forthcoming 'single European market' was averted last week at an emergency meeting of European Communities (EC) agriculture ministers in Brussels, after France, West Germany and Italy dropped their bans on the import of British beef. The bans were introduced on the grounds that public health might be at risk from the bovine spongiform encephalopathy (BSE) epidemic in British cattle — although British agriculture minister John Gummer had accused his European counterparts of acting for economic reasons. British exports of beef to France amount to over £150 million per year.

In return for the resumption of trade, Gummer had to accept that all exports of British beef carcasses to other member states must come from herds that have been free of BSE for at least two years. This is a climb-down for Gummer, who last year rejected West German calls for a similar 'certification' scheme.

Nevertheless, a spokeswoman for the British agriculture ministry delegation in Brussels said that the agreement was a

good one, as trade would be resumed.

The new controls mean that carcasses from about 7,500 herds (5 per cent of the UK total) cannot be exported to other member states, unless meat is de-boned and nervous and lymphatic tissues are removed.

The EC agriculture ministers' joint statement also notes that the European Commission is developing surveys to pick up cases of BSE, which was made a notifiable disease in the EC earlier this year. But John Wilesmith, who is leading the UK agriculture ministry's epidemiological work on BSE, says that surveillance in other member states where BSE may become a problem (see below) is less thorough than in the United Kingdom.

The Commission is also reviewing the processes used in manufacture of animal feed across the EC. Britain and the Netherlands have banned the use of ruminant feed containing ruminant protein, thought to be the cause of the BSE outbreak in British cattle, and EC-wide regulations are a possibility in future. But for the time being, the Commission says that animal feed regulations will be left to individual member states. **Peter Aldhous**

BSE FRANCE

The unexpected export?

Paris

FRENCH veterinary experts are worried that their farmers may be sitting on a time-bomb. The meal containing ground meat from scrapie-infected sheep, now implicated in the spread of bovine spongiform encephalopathy (BSE) in Britain, was imported for a short while into France and may trigger an epidemic after the four-year incubation period is over.

There has even been some speculation, sparked by a report in the British newspaper, *The Independent*, that cases have already occurred and French farmers are covering them up. The newspaper argues that as BSE has not been officially declared a contagious disease in France, farmers could not claim compensation if BSE cases were found and are thus under pressure to declare them as something else — rabies, for example — for which compensation is given.

This is improbable, said Jeanne Brugère-Picoux, head of the bovine pathology department at the Maison d'Alfort Veterinary College near Paris. "I do not think there is much risk of confusion in the clinical diagnosis of rabies and BSE", she said. "A cattle farmer would call a veterinarian if he was worried. He would not make a diagnosis himself".

And cattle suspected of having rabies have, by law, to be sent to the National

Veterinary and Foods Research Centre (CNEVA) at Nancy for post-mortem tests. Jean Blancou, head of the national rabies laboratory at CNEVA said that, since January this year, the brains of cattle that have died with unexplained neurological symptoms, but prove not to be rabid, are sent to Maison d'Alfort for BSE histology. "We have sent 53 brains this year, but none was BSE positive."

But Blancou admits that there are loopholes in the present screening procedures. CNEVA theoretically tests all cattle suspected of rabies. In practice, only farmers from the 40 departments where rabies is endemic send cattle to Nancy for tests.

Although agreeing the system is less than perfect in departments where rabies does not occur, Blancou says that he does not think there have been any cases of BSE. To block the loophole, the Ministry of Agriculture will have ready in a few weeks a decree declaring BSE a recognized disease, so that farmers will receive compensation.

Although no cases of BSE have been reported in France, Brugère-Picoux says there is still a question of whether French cattle have been infected. Since the disease takes over four years to develop, "we will have to wait until 1992 to find out", she said. "We imported British meal, even when Britain banned ruminant-based feed

ANIMAL LIBERATION MOVEMENT —

Terror replaces debate

London

IN a disturbing development, two British animal researchers narrowly escaped serious injury last week when their cars were destroyed in separate car bomb attacks. On Wednesday, Margaret Baskerville, a veterinary officer at the Ministry of Defence's chemical defence research establishment at Porton Down, in Wiltshire, was the intended victim. On Sunday, the target was Max Headley, a neurophysiologist from the University of Bristol's physiology department. The Bristol blast partially severed the finger of a 13-month old baby boy, embedding shrapnel in his back.

There is speculation that the attacks could be linked to the explosion which destroyed a bar in the University of Bristol's Senate House administrative building last year — a crime which has remained unsolved. It is thought that a splinter group from the militant Animal Liberation Front (ALF) could be responsible, and a local television company has been contacted by a caller, allegedly an ALF member, who claimed responsibility for the Bristol bombing. Chief inspector Bryan Saunders, from Avon and Somerset police, says detectives are "giving some credence" to the call. ALF's previous activities include assaults on laboratories to 'liberate' experimental animals, and incendiary attacks on department stores selling furs, but its leadership has rejected direct violent action against people.

The attacks have been condemned by police, researchers and animal rights pressure groups. The use of plastic explosives (traces of which were found in the wreckage of Baskerville's car) suggests that the group responsible had intended to kill or maim. The bomb under Headley's car was placed under the front passenger footwell. Police say that any passenger would probably have been killed.

Avon and Somerset police have been accused of complacency over the Bristol bombing. A neighbour spotted a suspicious package under Headley's car on Saturday, and warned the police. Saunders says a check was carried out against records to confirm that the car was owned by a resident of the street in which it was parked, but admits "an officer should have gone to examine the vehicle". **Peter Aldhous**

for ruminants in 1988." The remaining issue, says Brugère-Picoux, is whether it is safe to eat infected meat. "We do not know the illness well enough to be scared", she said. "But if the risk is small, even negligible, it cannot be ignored. Transmission from cattle to man has not been proved, but it has not been disproved either. We need to be careful and vigilant. I eat beef, but I would not eat calves' brains". **Peter Coles**

DRIFTNET FISHING

Net disagreement

Washington

THE news that the fleet of North Korean fishing boats arrested last month by the Soviet Union for illegal driftnet fishing close to the Kamchatka Peninsula was actually crewed by Japanese fishermen has caused a serious diplomatic incident. And it has strengthened US demands for a total international ban on the use of driftnet fishing. Even though Japan has no diplomatic relations with North Korea, Japanese driftnet fishermen made a deal with a group of North Korean fishermen in the hope of avoiding the high charges levied by the Soviet Union on Japanese boats fishing for salmon in the area.

To some US congressmen the incident is evidence of the how far fishermen will go to bend the rules governing the use of driftnets. These multiple 5–10 km-long fine nylon structures are being blamed for destroying Pacific fisheries and indiscriminately stripping the seas of all living creatures. In response the US Senate Commerce committee reauthorized the Magnuson Act, which originally established the 200-mile Exclusive Economic Zone and is reviewed every five years, with an additional clause intended to secure a permanent ban on driftnet use on the high seas. The act has already passed the House of Representatives, and is expected to go to the Senate later this year.

No profit came to the Japanese fishermen from their deal with the North Koreans. Although, according to the Asahi Shinbun newspaper, the fishermen had hoped to make money by gaining access to the North Korean quota of Soviet salmon in exchange for allowing the North Koreans use of Japanese boats and technical guidance. They discovered, on their arrest by Soviet vessels, that North Korea actually had no salmon quota under the Soviet-North Korean fishing agreement. A total of 169 Japanese fishermen on 12 boats were arrested.

The North Korean incident and the subsequent Senate action comes as an embarrassment to the Japanese government. Last winter Japan adopted a United Nations General Assembly resolution intended to impose a moratorium on the use of massive driftnets from 30 June 1992, except in cases where it can be proven that an appropriate resources management scheme will be effective. A moratorium would be bad news for Japan, which operates the world's largest fleet of driftnet ships — a total of 900 ships generating an income of ¥48 thousand million (\$300 million) a year.

To avoid the moratorium, the Japan Fisheries Agency has just begun a large-scale research effort to see how seriously driftnet fishing affects the effective con-

servation of marine creatures. At risk are not only commercial fish stocks (see *Nature* 342, 9; 2 November 1989), but also other species of fish, marine mammals and sea birds. The research is being conducted with the cooperation of US and Canadian observers and an interim report is expected to be delivered later this month.

"We can't say how much impact driftnet fishing has on the marine ecosystem now. We know that dolphins, turtles, young whales, and others are killed by the massive driftnet, some of them are endangered species. But to get information on the magnitude of the impact and predict the future, we must wait for the result of

CZECHOSLOVAKIA

Students terrorized

Pilsen

VIETNAMESE students in Czechoslovakia say that they fear for their lives as increasing freedom has brought racism into the open. One group of students, from the electrical engineering college in Pilsen, wrote to President Vaclav Havel asking him to help protect them from skinheads who have terrorized them and attacked gypsies and Vietnamese workers. Havel responded by criticizing the "dark forces" in Czechoslovakia — meaning former Communists or other agitators intent on causing anarchy. Violence has increased everywhere, especially since the government emptied the prisons in an amnesty that was apparently not restricted to political prisoners.

University rector Jiri Holenda said that the Vietnamese students are right to be afraid, even though the violence has mostly



been directed at Vietnamese workers, who have a reputation for being active in the black market.

According to Radio Free Europe, there are more than 35,000 Vietnamese workers in Czechoslovakia. Their labour was made available by the Vietnamese government in repayment for Czechoslovak aid during the Vietnam War.

Steven Dickman

this research," said Susan Smith, a researcher at the US National Marine Fisheries Service, which is collaborating on the project.

US congressional action comes far too soon for Japan, which would like to wait for more research data before considering its response to the UN moratorium. Japanese government officials say that increasing pressure for a total ban on driftnet fishing may bring more embarrassment for Japan. "Drastic pressure will produce a vicious circle — the stricter the regulations, the cleverer the loopholes [that will be found to avoid them]. This incident of 'piracy', which stains Japanese efforts to deal with the moratorium, is unlikely to be the last," predicted one government official.

Shigeko Segawa

COCOM REFORMS

Easier access

Paris

THE 17-nation Coordinating Committee on Multilateral Export Controls (CoCom) has agreed to redefine restrictions on the sale of technology to Warsaw Pact countries. The changes, adopted at a two-day meeting here last week, are the most significant since CoCom was set up in 1949.

The move comes in response to political changes in Eastern Europe and pressure from Western industry. The reforms will mean that the current list of sensitive technologies will be replaced by a new "core list" by the end of this year. Fewer items will be controlled, but security on these will be much tighter.

The new core list will have eight categories ranging from electronic systems, advanced materials and lasers to avionics and computers. An industrial list of controlled technologies has also been cut dramatically — 30 headings have been removed out of 116.

In future, sales of computers operating at 250 megabytes per second will be allowed and there will be easier access to those operating at over 1,000 megabytes per second. Restrictions will also be eased in telecommunications technology and sales of precision machine tools.

Eastern bloc countries will not be equally affected by the new rules, while almost no restrictions will be applied to East Germany, the Soviet Union will benefit least. Preferential treatment is to be given to Poland, Hungary and Czechoslovakia.

Despite the freeing of restrictions, some technologies will continue to be carefully guarded. Last week US and British governments stepped in to veto proposals by two competing telecommunications firms — US West Inc and British Telecom — to lay fibre optics cables stretching from Japan to Western Europe across the Soviet Union. The technology has military applications and is used in missile control.

Peter Coles

Museum's plans beyond belief

SIR—I read with disbelief your recent leading article (*Nature* 345, 1; 1990) and letters from colleagues regarding the British Natural History Museum's corporate plan announced on 23 April.

Museums have two important functions and the great ones do both: (1) museums are centres for empirical research on the material evidence of diversity and development of a subject, be it art or science, and (2) museums are centres for exhibition of evidence, enriching information available in books, films and classrooms to substantiate a grander world beyond access to the average reader/viewer/student. Research develops the meaning of this evidence in its widest possible context, and exhibition substantiates the credibility of book and classroom learning for the public at large.

I have worked regularly in the Natural History Museum for many years, long enough to have seen it change. The greatest change has been in the exhibition programme where exhibits increasingly duplicate information available in books, films, classrooms and television — less often featuring specimens and I-can-see-it evidence of diversity and development in natural history. Exhibits are expensively and extravagantly dominated by action and colour befitting entertainment centres rather than an educational institutions.

If research and exhibition cannot be brought into better balance, sound corporate management suggests research collections might be given away (or better sold) to speed convergence with Disneyland. Americans and Germans and Japanese have always coveted the Natural History Museum's research collections (and the extraordinary natural history learned from these) so there shouldn't be a problem finding buyers. Students can always travel overseas if they really want to learn about nature.

PHILIP D. GINGERICH

University of Michigan,
Museum of Paleontology,
Ann Arbor, Michigan 48109-1079, USA

SIR—In a recent article about the cuts in scientific posts at the Natural History Museum¹ it was reported that one of us (K.A.J.) had “condemned the cuts as ‘ludicrous’”. This terse comment was not intended to imply a judgement on each and every one of the cuts. In order to capture the intended nuance it needs to be amplified to read: “condemned the financial situation which has led to the cuts as ‘ludicrous’”.

At a time when there is a widespread concern for the loss of biodiversity, it has come to be realized that perhaps 90 per cent of species remain unknown to science. In order to remedy this, it has

been suggested that the number of taxonomists needs to be increased about twenty-five-fold². But systematic biology is being run down³: it is fast disappearing from institutions of higher education as it ceases to attract funding^{4,5}, and the universities are not in a position to appoint taxonomists if they are to fulfil their commitment to other, more costly, areas of biology.

We believe that the crisis confronting systematic biology (including taxonomy, palaeontology and comparative morphology) in Britain is now so grave that new initiatives are called for. Rather than just deploring the cuts, we offer the following positive proposals.

(1) Pursue the suggestion⁶ that the Linnean Society — the one national learned society serving the interests of the whole of biology — might set up investment funds to establish research fellowships which should, at least initially, give priority to systematic biology. Can funds be generated by an appeal to commercial organizations? A large pump-priming grant from central government would set an example, and indicate that it has received the message that basic taxonomic studies are an essential prerequisite to responsible management of the environment.

(2) The Natural Environment Research Council (NERC) should establish a committee to support systematic biology. Furthermore, NERC needs to reformulate its guidelines to allow the granting of funds for salaries for research workers in systematic biology who do not already hold salaried posts. Most research in these fields does not require expensive equipment: the impediment is the lack of salaries.

(3) The authorities should be persuaded to provide the resources necessary to allow the Natural History Museum to maintain its position as the international centre of excellence in systematic biology.

K. A. JOYSEY, J. A. CLACK, M. I. COATES,
R. H. L. DISNEY, W. A. FOSTER, A. E. FRIDAY,
A. M. LISTER & R. C. PREECE

University Museum of Zoology,
Downing Street,
Cambridge CB2 3EJ, UK

1. Gee, H. *Nature* 345, 4 (1990).
2. Holden, C. *Science* 246, 754–756 (1989).
3. Black, C.C. (Chairman) *Natn. Sci. Fdn., Natn. Sci. Board, Natn. Rep.* 89–171, 1–19 (1989).
4. Disney, R. H. L. *Linnean* 6(1), 12–14 (1990).
5. Ehrenfeld, D. *Conservation Biol.* 3, 415 (1989).
6. Erzinçoglu, Y. Z. & Fraser, N. C. *Linnean* 6(2), 9–10 (1990).

SIR—When I suggested two years ago (*Nature* 333, 292; 1988) that “the current performance behind the turnstiles and showcases of this national institution turned scientific Disneyland in relation to its past traditions and promises [needs]

more public scrutiny”, it did not occur to me that the next director of the British Museum (Natural History) might take the comparison seriously and decide he could get away with it. Even six months ago, he was still professing that “the Natural History Museum will move on to ensure that the museum retains its position as one of the world's great research-based museums of natural history. Its magnificent collections and the curatorial expertise of its scientific staff will continue to provide the foundation for its science. . .” (Neil Chalmers, *Times*, 29 November 1989). It may therefore be of interest to consider the implications of the recent cuts.

Last autumn, I asked my MP, Alick Buchanan-Smith, to take up with the minister the declining standard of service in the subdepartment of ornithology, which suffered severely in an earlier round of cuts, after I had made a journey down from Scotland to pick up some documents deposited in the subdepartment and found that they were not ready. Richard Luce replied on 6 November that “in the last week approval has been given for the appointment of an additional junior curator and I am sure that this will help the Department to deal with enquiries”. When I returned a couple of weeks ago I was therefore surprised to find that they were still so short-staffed that they had to close at lunchtime, and discovered that they had apparently attempted to fill this vacancy by advertising in the local newspaper, whereupon the only potentially suitable applicant had rapidly decided to accept a more attractive offer elsewhere.

I was concerned to find that my previous complaints of a declining standard of service in a subdepartment affected by past cuts have been misrepresented as criticism of the surviving staff, who are continuing to do their best under deteriorating conditions. I do not wish to criticize them. But it may still may be questioned how long they will be able to maintain even the present standard if this is the way in which it is proposed to try and replace the doctors of philosophy apparently declared redundant so that the museum can masquerade as expert in more fashionable fields where it has previously made little contribution. Although it is interesting to observe that the staff in the main museum at South Kensington are at last beginning to show some solidarity in resisting further cuts there, one could have wished that together with our universities they had shown a little more unanimity at the start of the process of attrition, instead of allowing it to proceed to the lengths that you have now described.

W. R. P. BOURNE

Department of Zoology,
University of Aberdeen,
Tillydrone Avenue,
Aberdeen AB9 2TN, UK

The museum that has to change

Walter Bodmer

The new corporate plan for the British Natural History Museum, which entails loss of scientific posts, has been widely criticized. But tough decisions had to be made to safeguard the museum's future.

THE widespread and high esteem for the Natural History Museum (NHM) is reflected in the enormous response we have received to our corporate plan. This level of interest in our financial situation and its implications lends strong support to the case for increased resources for taxonomical research.

The NHM has four interrelated functions.

(1) It must maintain and act as curator for its collection of over 67 million specimens as well as for its library, the largest natural history library in the world, and its remarkable collection of art on paper, the third largest in Britain. This extraordinary collection of materials must be maintained in a satisfactory state and made available for those who need it for their research, whether from the museum or elsewhere.

(2) The museum supports a major research programme based on its collections, with taxonomy as its central theme. It is the main home for such research in Britain, research which cannot be carried out in a comparable way in other institutions, including the universities. Taxonomy is the science of classification of living organisms and, with its evolutionary basis, provides the underpinning to almost all biological research. It is as relevant to fundamental understanding of the evolutionary process as it is to identifying the insects which are transmitters of human or animal diseases. In addition, of course, the museum has a unique collection of minerals which forms the basis for its research in mineralogy and now also includes the important collections in the former Geological Museum. It is also the centre of an international scientific network. Every working day it receives an average of 100 scientific visitors, and every year it lends some 70,000 items from its collections to other scientists around the world.

(3) The museum must exhibit its collections, at least in part, and explain the associated science at all levels. This is the aspect of the museum that is most familiar to the general public, who are mostly not aware of the size of the collections and the range of research that is carried on behind the exhibition halls. The exhibitions are especially valuable for school children, for whom they provide a complementary stimulus to the science learned at school. This is now particularly important following the introduction in Britain of the national curriculum with its focus on

environmental, health and Earth sciences, all of which are integral to the museum's activities.

(4) Lastly there is the obligation to maintain the splendid Victorian Waterhouse building, and the many other buildings which house the collections and research activities, as well as the museum at Tring. There is the challenge of fitting new and attractive exhibitions into the Waterhouse building without detracting

The essence of our financial problem is that the government's 'grant in aid' will not compensate adequately for inflation. . . . We have no alternative but to live within our means.

from its architectural heritage. The main building is in a relatively good condition; but on the other hand, we have to face heavy capital expenditure in replacing our outlying store at Ruislip. Building and building maintenance costs are provided separately by the government, and we can only do that for which we are funded.

Until 1987 the museum was funded by the Department of Education and Science, through the 'science vote' that pays for the research councils, on the basis of its role as a major research organization. But there were many people at the time who argued that the science vote was not the appropriate source of support for activities such as the maintenance of the museum's collections and exhibitions, and the upkeep of its historic building. For this reason the Office of Arts and Libraries, which looks after the other major national museums and galleries, seemed a better home. The NHM is unique in its mixture of scientific research and the more traditional museum activities and, notwithstanding our present difficulties, has found a welcome place in the Office of Arts and Libraries. This change has also provided the museum with new opportunities for additional research support from the research council system.

The corporate plan is not a detailed scientific document, but is an outline scheme that is essential for future planning and budgeting. Priorities have to be set for overall activities in research and for exhibitions, the building programme, fund raising and the organization of general management. Without such a forward plan the case cannot even be made for the current level of the

museum's resources, let alone for any expansion. The need for such a plan is still there, even if income is rising. The most dangerous assumption to make is that no changes are needed because all has worked for the best in the past. Science is advancing rapidly in all areas, approaches to the design and construction of exhibitions are changing radically, and the public's needs and desires are altering too. Not to take account of these changing circumstances would surely be to turn the museum into a dinosaur like the ones it so successfully exhibits.

Large areas of the exhibition galleries need to be renovated and the exhibitions themselves upgraded. This is both a costly and a challenging exercise. The exhibitions must educate and entertain in a modern idiom. If we do not attract an audience, and perhaps learn to some extent from the wonders of Disneyland, then there is no use in putting on an exhibition, however academically excellent it might be. There is also always the need to display part of our collections, while still making them all available for both the professional and the amateur research communities.

Research work at the museum is being reorganized into six main programmes that emphasize the particular contribution the museum's collections and taxonomic expertise can make to contemporary concerns. These are: biodiversity (which can include almost any area of taxonomic and evolutionary research); environmental quality; living resources; mineral resources; human health; and human origins. Their interdisciplinary nature will enhance collaboration between different departments within the museum, and with other national and international organizations. Research in all these areas will be taxonomically based and provides enormous challenges, both basic and applied. While accepting the need for the highest quality of basic research, it surely must also be sensible to concentrate taxonomic activity on potentially useful and applicable areas. Appropriately, given concerns about the environment which range over all the six research programmes, this is where more resources for research are available.

There are new scientific challenges for the museum's research that come from the revolution in our understanding of the genetic material and our ability to manipulate it. Some of the collections can now

provide a source of the ultimate information on which taxonomy must be based, namely the DNA sequence itself. Neither can the revolution in information technology be ignored by the museum. In order to develop such new and exciting areas, there is no alternative to redeploying resources, certainly within a finite and even within a growing budget. Resources are also needed to attract top-quality scientists who will only come if they can get the support that they need to exploit new areas of research using the collections. The museum has an unusually high percentage of its staff on permanent appointments. But the vitality of a research institution depends to some extent on a succession of bright young people, some of whom will stay for longer. So there is a need to create more flexibility in scientific appointments.

It would be impossible to carry out effective research on all the groups of organisms in the museum's collections even if our resources for research were doubled or trebled. There is a requirement to maintain all the collections and make them available to research workers worldwide, even if they are not the subject of specific research in the museum. Curation, in this sense, is an important and challenging task that involves developing detailed knowledge of the objects being cared for under the supervision of senior and experienced scientific staff. Such a directed programme for the maintenance of the collections is essential if the museum is to meet its obligation for their proper care.

The essence of our financial problem is that the government's 'grant in aid' will not compensate adequately for inflation. Obviously, even a small difference between the two is enough to lead to a serious financial deficit over a period of five years. The trustees would be grossly irresponsible if they allowed such a deficit to accumulate. We have no alternative but to live within our means. At the same time we must strongly argue our case for increased resources from the government and make strenuous efforts to increase income from other sources. That is one of the main aims of the corporate plan. The cuts now being proposed in order to remain within our budget are severe, but less so than some of those that have in the past been faced by the research councils with which we have the closest affinity.

Posts at the museum have been lost continuously over the past five to seven years through natural wastage. But this cannot continue, because the result is haphazard erosion of the museum's activities making future planning impossible. If we do not take action now, we shall soon face a situation in which the government's 'grant in aid' is used entirely for salaries — an intolerable position, which leaves no scope

for flexibility for the development of our research.

There is no money to redeploy from elsewhere and so posts must be cut if we are to balance our budget. Cuts have already been sustained in many other areas of the museum's activities and we cannot now sustain further curtailment of our exhibition programme, which in any case is substantially supported by non-governmental sources. Most of this money, which comes from private sources, cannot be used to substitute for a shortfall in the government's provision for the research on our collections. We must also maintain our support for income-generating activities, otherwise our financial crisis would be even greater. Under the present circumstances there is, therefore, nowhere else where further savings can be achieved other than our scientific programme.

The scientific programme of the museum is subject to stringent, periodic review by distinguished external assessors acting together with scientists of comparable distinction who are members of the board of trustees. It is against this background that the director and his senior staff must assess research priorities while taking into account not only the museum's own strengths but also those of comparable institutions in Britain and elsewhere. There is no doubt that, whatever areas were curtailed, they would be the ones subject to the most forceful complaints. This is exactly the situation the museum now finds itself in; criticism concerning areas of research which are being cut has come almost entirely from individuals who work in those areas.

The NHM has been more active and successful than most of the other national museums in its efforts to raise money from non-governmental sources. Its development trust set a target of raising £5,000,000 within five years but has already reached commitments of nearly £4,000,000 within the first year of its activities. This has been achieved through the dedicated efforts of distinguished and busy trustees and other members of our fund-raising committee. The fund-raising has focused on that area judged to be most likely to be supported by private donations and sponsorship, namely the exhibition programme. The industries and individuals that have supported us share our concern for a population that understands the science of natural history and its importance for our environment, and for our future health and welfare. We will, of course, also present the case for private support for our scientific programme, for example through the endowment of research posts. But our greatest hope for extra research support must lie in successful competition for grants and contracts within Britain and the European Community, making the case for the import-

ance of taxonomically based research.

There is no question of any money being diverted from research to exhibitions. Rather, it is the case that if our efforts at raising funds for exhibitions had not been so successful, then the situation for the support of our research activities would have been truly catastrophic. The public and sponsors do not wish to give money for deficiencies in government funding. Unless the government reimburses us adequately for inflation then our situation will be even worse than that predicted in the corporate plan, and no doubt the same would be true for many other government-supported activities.

The museum's trustees, who together have a range of expertise across science industry and commerce, have been closely involved in the development of the corporate plan which it is their responsibility ultimately to approve. We are unanimous in our support for the director and his staff in producing an imaginative and challenging plan that deals both with the need for restructuring some of the museum's activities to sustain its excellence in all directions, and with the challenge of living within a limited and possibly declining budget. It is clear that not all of those who have written to us expressing their concern for the future of the museum have had the opportunity, or sometimes perhaps the time or the inclination, to read the corporate plan carefully. Had they done so, we are sure that rather than criticize the museum they would wish to join us in making our case as strongly as possible for increased resources from the government. We, of course, have the greatest possible interest in promoting our case and have, for example, urged that the funds we raise through our development trust be matched by the government as a sign of its commitment to supporting the museum's enterprise in raising extra funding.

Beyond this, we continue to make the case for the importance of taxonomically based research and the museum's unique role, especially in the light of current concern about environmental changes. The increased funding provided for environmental research through the research councils and other government departments must make a contribution to the support of the museum's research programme over and above the core funding which we receive from the Office of Arts and Libraries. These are the cases we need to make, together with all those who are concerned for the future wellbeing of the NHM, so that we can build up our strength in high priority areas of taxonomical research. □

Sir Walter Bodmer is director of research at the Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, UK, and chairman of the trustees of The Natural History Museum.

Nuclear physics for arms control

Two US and Soviet pressure groups have joined forces to produce a study of future goals in strategic arms control which is also an invaluable guide to the technical problems of verification.

THE usually combative demeanour of the Federation of American Scientists (FAS) exuded contentment at the modest ceremony last week at which it launched its latest study, with the Moscow-based Committee of Soviet Scientists (CSS), of the prospects for reducing stockpiles of nuclear weapons. True, only a few days earlier, Presidents George Bush and Mikhail Gorbachev had said they would sign a strategic arms control agreement before the year is out, which is consistent with what both pressure groups have been demanding, but the proposed treaty is likely to fall short of the 50 per cent reduction foreseen eight years ago, let alone of the 2,000 warhead limit for superpowers advocated by the new study. So why the contentment?

To be fair, Frank von Hippel of Princeton, co-editor with Roald Sagdeev of the Moscow Space Research Institute of the volume *Reversing the Arms Race* (Gordon & Breach, New York; in press), made it plain that "START 1 should be quickly followed by START2", and it has been agreed that work should begin on the more ambitious treaty before the first is ratified (perhaps by the end of 1991). But nobody seems to think it feasible that the first treaty might be toughened before it is signed. Is it just possible that the arms control pressure groups have come to share with weapons manufacturers the sense of loss that an interesting problem has been snatched away?

That near-blasphemy is almost sustained by the fine print in this book, the first in a series under the title "Science and global security", whose appendices and footnotes deal absorbingly with the considerable technical problems arising in the course of verifying compliance with treaties that limit strategic arms. The fine print provides some intriguing information about the arrangements made for verifying compliance with the INF treaty restricting missiles of intermediate range. In passing, it also throws light on the problems of detecting forbidden objects and substances at civilian airports.

One has to marvel at the painstaking work that has gone into this fine print — and also at the ingenuity. Who, for example, would have thought of sealing objects that must not be tampered with by roughly wrapping a bundle of optical fibres around them and recording the almost irreproducible pattern of light obtained by shining light down a few selected fibres?

The basic issue is that of detecting a nuclear warhead, or of telling whether a missile is thus equipped, by remote sensing alone. (The standard provisions of the INF treaty, being followed in START1, are that a missile owner's word may ordinarily be verified only instrumentally, but that each party to the treaty is allowed a quota of direct inspections 'on challenge' each year.)

Uranium and plutonium bombs present different problems. Although both materials emit neutrons (from spontaneous fission) and γ -rays (from fission, fission products and the products of radioactive decay), neutron detection is likely to be effective only for plutonium (which has the higher spontaneous fission rate). The study concludes that γ -detection is to be preferred for both, relying on the characteristic 1.001 MeV γ -ray from ^{234}Pa (protoactinium-234) and the 0.662 γ -ray from ^{241}Am (americium-241) in the decay chains of ^{238}U and ^{241}Pu respectively. Given time (to reduce background) and sufficiently sensitive detectors, these γ -rays may be detectable at ranges of several metres, even with portable detectors.

The snag is that neither of the characteristic γ -rays comes from the isotopes from which bombs are made (^{235}U and ^{239}Pu respectively). Almost lovingly, there is an appendix describing how bomb-makers might plan to avoid the detection of their warheads by various devices — purifying weapons-grade Pu by separation of tell-tale isotopes (the high spontaneous fission rate of ^{240}Pu , for example, is a potential giveaway.) Similarly, the remote detection of warheads made from ^{235}U which are not equipped with a uranium tamper (tungsten is sometimes used instead) would be difficult.

So why not more active sensing devices? Radiography is a candidate, based on the relatively high stopping-power of nuclear explosives, but would probably require portable linear accelerators to be sensitive. (Some manufacturers' names are quoted.) By way of illustration, the report includes a γ -ray radiograph of a North American motor car which shows the engine to be in the front, where it belongs.

Neutron activation, which might have been thought the obvious candidate, requires surprisingly intense sources of 14 MeV neutrons to yield quick results by neutron detection (but there seems to be a future in the detection of fission-product γ -rays). This, of course, is where warhead detection and aircraft security may

make common cause; civil aviation authorities hope that neutron activation machines can detect hydrogen-rich organic explosives, but the point has yet to be proved.

In this connection as in others, the new report suggests that, like nuclear physics itself, the refinement of techniques for warhead detection will hardly ever be complete. What FAS and CSS have between them done is to produce a *vade mecum* for the teams of nuclear physicists who will for decades hence be camped outside the gates of Soviet and US weapons plants, counting warheads as they leave and enter, and a prospectus for research by those who stay at home.

Even the START1 treaty will require some means of verifying that information provided about sea-launched cruise missiles is correct. The essential difficulty is that the proposed treaty will limit the numbers of strategic warheads deployed by the Soviet Union and the United States, but that numbers of cruise missiles (some of which may carry conventional warheads) will not be limited. The problem does not arise with air-launched missiles, which will effectively be limited by the restrictions to be placed on the numbers of carriers (bomber aircraft) which are deployed.

A Soviet proposal that nuclear warheads might be detected from helicopters flying near ships equipped with nuclear warheads is floated in the new study, but the measurements reported (a decrease of neutron-count with distance) do not seem compelling. There seems nothing for it but on-board inspection — in which connection a US team reports the remote detection in the forward missile tube of a Soviet cruiser sailing in the Black Sea of a ^{235}U warhead at the tip of a cruise missile.

But plainly, on the evidence of this report, it is clear that sea-launched cruise missiles will be a headache for those who administer START1. Each signatory will have to provide the other with a list of how many nuclear warheads are carried on what ships, and the object of verification will be to ensure that the whole inventory remains what it is supposed to be. That will entail not merely the inspection of vessels in port and at sea, but also the monitoring of missiles as they are loaded and unloaded at predesignated ports. On the evidence of this excellent study, it is not surprising that these weapons have been a stumbling block in the negotiation of START1.

John Maddox

Fast-acting slow viruses

Malcolm A. Martin

In the past seven years there has been a virtual explosion of new data about the human immunodeficiency viruses (HIVs) and their lentivirus relatives. Progress in molecular biology has been particularly impressive — genomes have been sequenced, viral-encoded proteins have been identified, novel regulatory proteins and *cis*-acting elements have been elegantly dissected and, in a few instances, genetically engineered viral gene products have been inoculated into animals and man in attempts to elicit protective immunity. A considerable impediment to advances of equal importance in HIV biology and pathogenesis has been the lack of a tractable animal model. As a result, interest has shifted to the simian immunodeficiency viruses (SIVs) to provide answers to questions involving the life cycle of primate lentiviruses *in vivo*. Of particular note have been reports of molecular clones of SIV which direct the synthesis of replication-competent progeny virions. Unfortunately, none of these generate a virus capable of inducing disease in inoculated animals. Two reports, however, one by Kestler *et al.* (Desrosiers's group) in *Science*¹ and the other by Dewhurst *et al.* (Fultz's group) on page 636 of this issue², describe the development of fatal disease in monkeys exposed to virus derived from new SIV clones. Experiments *in vivo* can now proceed full steam to evaluate the determinants of pathogenicity, the genomic variability, the role *in vivo* of some lentiviral accessory genes (for example, *vif*, *vpr*, *vpx* and *nef*) and potentially useful antiviral agents or vaccines.

SIV variant

Simian immunodeficiency virus induces a syndrome in experimentally infected rhesus macaques that is remarkably similar to human AIDS. The animals develop an immunodeficiency state characterized by a loss of lymphocytes bearing the CD4 antigen, opportunistic infections, diarrhoea with severe weight loss, and central nervous system disease; most animals die between one and three years after infection. Kestler *et al.*¹ used virus derived from a previously described SIV_{MAC} clone (SIV_{MAC-239}) which replicates efficiently in peripheral blood mononuclear cells (PBMCs) of rhesus macaques. They observed a typical immunodeficiency syndrome and death in five of eleven monkeys during the first year of infection. The affected animals lost a significant amount of weight, became immunodeficient, and died with disseminated viral, bacterial or *Pneumocystis* infections.

Fultz and her colleagues previously

identified an extremely virulent variant of SIV which, in contrast to SIV_{MAC}, causes an acute lethal disease in pig-tailed macaques as well as in other monkeys, with deaths occurring six to eight days after infection³. The variant, called SIV_{SMM-PBj14}, arose spontaneously in a pig-tailed macaque originally infected with SIV isolated from a sooty mangabey monkey (SIV_{SMM}). The pig-tailed macaques infected with SIV_{SMM-PBj14} developed a profuse, bloody mucoid diarrhoea during the first week after infection and died one to three days later from severe depletion of body fluids and electrolytes. SIV_{SMM-PBj14} differed from its SIV_{SMM} parent in several important ways: it had a wider host range (chimpanzee PBMCs and several continuous human T-lymphocyte lines), was cytopathic for sooty mangabey monkey PBMCs, and could not be neutralized *in vitro* with antibodies directed against SIV_{SMM}. Because of the fulminant and extremely rapid nature of SIV_{SMM-PBj14}-induced disease and its primary effect on the gastrointestinal tract, the report by Fultz *et al.* was initially viewed with scepticism in some circles because the syndrome was more typical of enteric pathogens than of lentiviruses.

Definitive proof that SIV_{SMM-PBj14} causes this rapidly lethal disease is now established from the work of Dewhurst *et al.*², who were able to generate fatal infections of pig-tailed macaques with virus derived from molecular clones. Three of six pig-tailed macaques infected with cloned SIV_{SMM-PBj14} died eight days after infection; two animals succumbed on days 49 and 55 and one is still alive seven months later. Sequence analysis indicates, as expected, that SIV_{SMM-PBj14} is closely related to SIV_{SMM} and shares less polynucleotide sequence homology with other SIVs and HIVs. Of note is a 23-base-pair duplication encompassing the single NF-κB enhancer element previously identified in the long terminal repeats of other SIVs.

What were the pathological findings in monkeys dying from this unusually fulminant and non-'lenti' (that is, rapid) lentivirus-induced disease? Philip Zack and colleagues (US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland) have infected juvenile rhesus, cynomolgus and pig-tailed macaques with SIV_{SMM-PBj14} and observed the rapidly fatal syndrome in all three species (P. Zack, personal communication). At autopsy, the most prominent feature was a two- to tenfold increase in the mass of many lymphoid organs; the spleen, mesenteric lymph nodes and the gut-associated lymphoid tissue (GALT) were the most severely affected. Diffuse

infiltrates of lymphocytes, consisting of up to 50 per cent lymphoblasts, were present in perfollicular areas. In the intestine, multi-focal, patchy blunting of villi was seen in conjunction with hyperplasia of crypt epithelium; little if any ulceration of the epithelial layer was apparent.

According to Zack, high levels of virus expression are the hallmark of acute SIV_{SMM-PBj14}-induced disease. From affected lymphoid tissue, 10⁴–10⁵ infectious virions could be recovered. Viral antigens were synthesized in 20–50 per cent of lymphocytes and syncytial cells located in the lamina propria; by contrast, no viral protein was detected in the intestinal epithelial cells. Budding virus particles could readily be seen in lymphoblasts, macrophages and syncytia in the GALT.

Immunological reaction

The pathological changes affecting the gastrointestinal tract seem to be more typical of an immunologically mediated reaction than of a necrotizing infectious enterocolitis. In fact, a picture of infiltrating lymphocytes associated with villus atrophy and crypt hyperplasia is more characteristic of graft-versus-host disease⁴. It is not generally appreciated that the normal human intestinal mucosa is rich in T lymphocytes, plasma cells and cells of the monocyte-macrophage lineage. Although most of this lymphoid tissue is in the lamina propria, lymphocytes also reside in the epithelial layer and may constitute up to 39 per cent of the cells in this part of the mucosa⁵. The migration of lymphocytes and lymphoblasts between these different intestinal compartments is extensive and tightly regulated. This mucosal lymphocyte population seems to play a large part in the renewal and differentiation of intestinal epithelial cells; alterations in intestinal T-cell function may, in fact, directly affect the proliferation of stem cells or early progenitor cells⁶.

With this as background, one can begin to speculate about how an SIV_{SMM-PBj14} infection might kill monkeys rapidly, and about which viral genes or elements could be responsible for its virulent phenotype. First, it is clear that SIV_{SMM-PBj14} induces an acute and fulminant viral disease rather than the typical protracted immunodeficiency syndrome elicited by other primate lentiviruses. Circulating lymphocytes become an early target of SIV_{SMM-PBj14} and support numerous rounds of vigorous and highly efficient virus replication. Subsequently, a tidal wave of infected lymphocytes enter and become sequestered in lymphoid tissues associated with the gastrointestinal tract. Indeed, SIV_{SMM-PBj14} can infect resting macaque lymphocytes *in vitro* and induce them to produce interleukin-2 (P. N. Fultz, personal communication). Assuming similar

events occur *in vivo*, it is not difficult to imagine how SIV_{SMM-PB14} might rapidly establish an extensive and spreading infection. But the migration of virus-producing lymphocytes to the GALT, and their apparent sequestration there, cannot yet be readily explained. Because SIV_{SMM-PB14} can induce cytokines such as interleukin-2 in cultured lymphocytes, it would not be far-fetched to propose that infected lymphocytes and monocyte-macrophage populations in lymphoid structures associated with the gastrointestinal tract secrete cytokines which influence the normal mononuclear-cell traffic in these tissues, or directly affect gastrointestinal physiology. One or more of the SIV_{SMM-PB14}-encoded proteins, such as an envelope protein that has undergone mutation, could elicit the 'inappropriate' production and/or release of such regulatory factors.

Monkeys infected with SIV_{SMM-PB14} apparently have high levels of circulating tumour necrosis factor (TNF), which may be another reflection of aberrant virus-induced cytokine release. Increased TNF production is also associated with HIV-1 infection of primary human monocytes⁷. The rapid clinical deterioration of SIV_{SMM-PB14}-infected macaques, which develop profuse bloody diarrhoea and an associated metabolic acidosis, is reminiscent of Gram-negative septicæmia, presumably also mediated by TNF (ref. 8).

Lentiviruses characteristically cause a mild primary syndrome, usually followed by a prolonged and unremitting secondary disease which may last for months to years. In man, acute HIV infection is associated with a 'mononucleosis-like' disease accompanied by cervical, occipital, axillary or even generalized lymphadenopathy during this early phase of infection in 75 per cent of cases⁹. Interestingly, diarrhoea has been reported in a third of recently infected patients¹⁰. The acute disease caused by feline immunodeficiency virus also results in pronounced generalized lymphadenopathy which centres on the gastrointestinal tract¹¹. Thus the disease induced in monkeys by SIV_{SMM-PB14} could represent an unusually aggressive and

highly aberrant primary lentivirus infection which efficiently and rapidly targets infected cells to lymphoid tissue in the digestive tract and results in death due to the loss of fluid and electrolytes.

The two new reports of infectious, disease-causing molecular clones of SIV also raise an interesting side issue pertaining to vaccine development. Fultz and her colleagues reported last year that asymptomatic sooty mangabey monkeys, previously infected with SIV_{SMM}, are completely protected from SIV_{SMM-PB14}-induced disease³. The two sero-positive monkeys challenged with SIV_{SMM-PB14} did not die or show any signs of secondary disease. Although Desrosiers and his

AIDS

Blocks on the viral exit

Jonathan Weber

Writing in News and Views nearly two years ago, David Baltimore¹ coined the term 'intracellular immunization' to describe the prospect of using a dominant negative mutant viral gene to interfere with the replication of wild-type virus. The most attractive early application of this approach to cell protection is against HIV infection — both because of the pandemic nature of this lethal virus, and the possibility of relatively easy access to its target, the CD4⁺ peripheral blood mononuclear cells. Three candidate HIV genes in which mutations can be made to give a dominant negative phenotype have already been described: *gag* mutants, which probably interfere with HIV replication at the level of virus assembly², and *tat* and *rev* transdominant mutants^{3,4}. Now, on page 625 of this issue Buonocore and Rose⁵ describe a novel approach to 'intracellular immunization' using a CD4 mutant that is retained by the endoplasmic reticulum and that prevents transport of the HIV envelope proteins to the cell surface.

Role of CD4

That the CD4 molecule is the cellular receptor for HIV has been known since 1984, and soluble recombinant CD4 (srCD4) neutralizes HIV infection of target cells *in vitro*, and probably *in vivo*. But less attention has been paid to the role of CD4 in the transport of HIV *env* proteins and the production of mature viral particles. Asjo and colleagues⁶ showed that CD4 receptor density on diverse monocytoid cell line clones from the U937 parental line was associated with the quantity of infectious virus produced; Hoxie *et al.*⁷, and subsequently Stevenson *et al.*⁸, demonstrated that down-regulation of CD4 post-HIV infection was associated with intracellular complexing of CD4 with HIV envelope protein. However, HIV

group have not carried out a similar experiment, they have isolated several molecular clones of SIV_{MAC}, in addition to the disease-producing SIV_{MAC-239} clone, which give rise to persistent virus infections; so far, however, the avirulent clones have failed to induce immunodeficiency in inoculated macaques. One wonders whether avirulent variants of HIV that can replicate efficiently *in vivo*, and elicit similar protective immunity in man, might also be isolated or even constructed in the laboratory. □

Malcolm A. Martin is in the Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892, USA.

env proteins can clearly be expressed in CD4⁺ cells, as shown by the Chinese hamster ovary cell line production of the HIV envelope glycoprotein gp120. The role of CD4 in the normal transport of gp120 requires further study.

Buonocore and Rose generated a mutant form of soluble CD4 (sCD4), which contains the sequence Lys-Asp-Glu-Leu (KDEL) at the C terminus; this sequence is a signal that can retain soluble proteins within the lumen of the endoplasmic reticulum⁹. Constructs of sCD4-KDEL were expressed in HeLa cells, with and without co-expression of gp120 and gp160. The sCD4-KDEL mutant prevented expression of gp120 on the cell surface, whereas normal sCD4 alone did not. In addition, the sCD4-KDEL mutant did not interfere with the normal surface expression of CD4 in the same cell. Using an assay for syncytium formation in HeLa CD4⁺ cells, Buonocore and Rose showed that sCD4-KDEL blocks syncytia formation after gp120/gp41 transfection. Theoretically, cells transfected with sCD4-KDEL should be protected from the consequences of HIV infection, while CD4 production and expression remains normal.

This approach to intracellular protection is elegant, and it avoids the use of dominant negative mutants based on viral genes which could lead to evasion of the block by further mutation. Any mutation of HIV *env* that prevents binding to sCD4-KDEL should reduce the binding of HIV to its receptor, and successful escape mutants from sCD4-KDEL should also be unable to infect CD4⁺ cells. But this study has not shown that wild-type HIV replication can be suppressed by sCD4-KDEL, nor that T cells transfected with sCD4-KDEL are functionally normal. It remains possible that sCD4-KDEL could become saturated by

1. Kestler, H. *et al.* *Science* **248**, 1109–1112 (1990).
2. Dewhurst, S., Embretson, J. E., Anderson, D. C., Mullins, J. I. & Fultz, P. N. *Nature* **345**, 636–640 (1990).
3. Fultz, P. N. *et al.* *Aids Res. hum. Retroviruses* **5**, 397–409 (1989).
4. McDonald, G. B. & Sale, G. E. in *The Pathology of Bone Marrow Transplantation* (eds Sale, G. E. & Shulman, H. M.) 77–103 (Year Book Medical, Chicago, 1984).
5. Ferguson, A. in *Immunological Aspects of the Liver and the Gastrointestinal Tract* (eds Ferguson, A. & MacSweeney, R. N. M.) 152 (MTP, Lancaster, 1967).
6. MacDonald, T. T. & Spencer, J. J. *exp. Med.* **167**, 1341–1349 (1988).
7. Merrill, J. E., Koyanagi, Y. & Chen, I. S. Y. *J. Virol.* **63**, 4404–4408 (1989).
8. Tracy, K. J. *et al.* *Science* **234**, 470–474 (1986).
9. Tindall, B., Cooper, D. A., Donovan, B. & Penny, R. in *Infectious Disease Clinics of North America — Medical Management of AIDS* (eds Sande, M. A. & Volberding, P. A.) 329–351 (W. B. Saunders, Philadelphia, 1988).
10. Cooper, D. A. *et al.* *Lancet* **i**, 537–540 (1985).
11. Yamamoto, J. K. *et al.* *Am. J. vet. Res.* **49**, 1246–1258 (1988).

gp120 within the endoplasmic reticulum, thereby by-passing the block; or that sCD4-KDEL escape mutants with lower affinity for CD4, but still retaining their tropism for CD4 cells, could be formed. These practical issues can be addressed only in an active HIV replication system.

Second generation therapy

Every week I watch AIDS patients deteriorate and waste away despite Zidovudine (ZDV) therapy. They urgently need novel therapies that act at different sites. Buonocore and Rose's report adds to the long list of potential AIDS therapies, and targets based on delivery of novel or mutated genes extend even beyond the mutant viral genes, into 'suicide' genes, or delivery of antisense RNA. If the reverse transcriptase inhibitors — both ZDV and the up-coming dideoxyinosine (DDI) — belong to the first generation of therapy, then the second generation will be drugs active at new sites. The entry of HIV is blocked by soluble recombinant CD4 *in vivo*, but at the cost of high doses of immunogenic recombinant protein; between 15 and 20 per cent of patients already have anti-sCD4 antibodies, and more will develop them after therapy¹⁰. The polysulphated polysaccharides — active *in vitro*, but seen as failures in a clinical trial because they did not act when administered orally — are now attracting renewed interest. They block entry of all HIV isolates and their principal toxicity (anti-coagulant activity) may be chemically separated from their anti-viral activity; at least one of these drugs may go back into clinical trials, this time through a more appropriate route of administration, as they are not absorbed when taken orally. Meanwhile, pharmacokinetic data on the HIV protease inhibitor peptides are eagerly awaited pending clinical studies.

It is unlikely that second-generation anti-retroviral therapy will be based on 'intracellular immunization'. But if the techniques can be refined, then transfection of an sCD4-KDEL construct into bone-marrow stem cells might be a very attractive third-generation option. □

Jonathan Weber is in the Infectious Diseases Unit, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK.

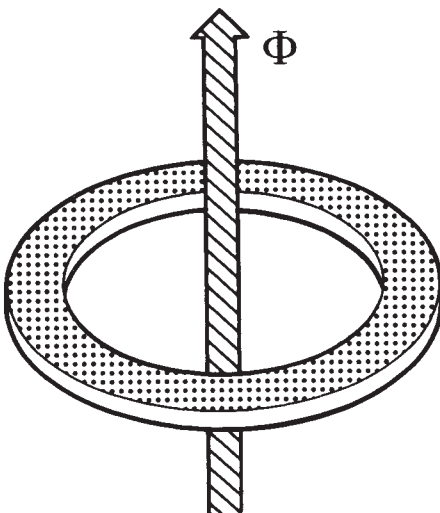
1. Baltimore, D. *Nature* **335**, 395–396 (1988).
2. Trono, D., Feinberg, M. & Baltimore, D. *Cell* **59**, 113–120 (1989).
3. Green, M., Ishino, M. & Loewenstein, P. *Cell* **58**, 215–233 (1989).
4. Malim, M., Bohlein, S., Hanber, J. & Cullen, B. *Cell* **58**, 205–214 (1989).
5. Buonocore, L. & Rose, J. K. *Nature* **345**, 625–628 (1990).
6. Asjo, B. *et al. Virology* **157**, 359–365 (1987).
7. Hoxie, J. *et al. Science* **229**, 1123–1127 (1986).
8. Stevenson, M. *et al. Cell* **53**, 483–496 (1988).
9. Munro, S. & Pelham, H.R.B. *Cell* **48**, 899–907 (1987).
10. Schooley, R. *et al. Ann. intern. Med.* **112**, 247–253 (1990).

Running around in circles

Yoseph Imry

It is well-known that currents that 'never decay' can flow in superconductors, but less familiar is the prediction¹ that such persistent currents can also be induced by magnetic fields in normal (non-superconducting) systems, with the proviso that they be small enough for quantum mechanics to apply. Levy *et al.*² now provide evidence for the existence of such currents, and moreover on the mesoscopic rather than microscopic scale.

Levy *et al.* have used electron-beam techniques to fabricate a system of 10⁷ square-shaped copper rings, 0.55 micrometres on a side. They employed a SQUID (superconducting quantum interference device) to measure the magnetic moment (proportional to the circulating currents) induced in the system at very low temperatures by a magnetic field applied



A conducting ring threaded by a magnetic flux Φ .

perpendicular to the plane of the rings. The magnetic signal was picked out from the background by harmonic generation techniques which rely on the specific nonlinear periodic response of the rings. The induced currents did not decay, at least on the timescale of the inverse frequency used in the experiment (a fraction of a second). Such times are huge on a microscopic scale, and are sufficient to qualify the currents as 'persistent'. The magnitude of the observed signal is consistent with theory within an order of magnitude, on the assumption that the signals from all rings add coherently³.

Despite theoretical expectations^{1,4-6}, there has been some debate about whether persistent currents can be maintained in a system of this sort. In classical systems, circulating currents must decay. But quantum mechanics permits a magnetic field to induce persistent dia-

magnetic moments associated with orbital electrons (in atoms and molecules, for example). In the experiments of Levy *et al.* these moments are associated with persistent currents that circulate around the rings. In macroscopic systems they may be expected to persist only when quantum effects still apply (in superconductivity, for example); so the existence of the effect in 'normal' mesoscopic systems was not generally accepted.

The circulating currents depend in a periodic way on the magnetic flux through the ring, being a manifestation of the quantum-mechanical Aharonov-Bohm effect⁷. This is a counter-intuitive phenomenon in which charged particles encircling a line of magnetic flux are influenced by the magnetic field even though they never pass directly through the flux line. The effect requires that the phase of the particles' wavefunction stays well defined for at least one complete revolution of a particle around the ring.

The persistent equilibrium current I in a ring threaded by a flux Φ (see figure) is proportional to $\partial J / \partial \Phi$, where J is the thermodynamic potential (for example, the ground-state energy for a closed system at zero temperature). Thus I is non-zero if J depends on Φ . The flux Φ induces a phase change $\phi = 2\pi\Phi/\Phi_0$ in the wavefunction of an electron that circulates once around the ring, so I is a periodic function^{8,9} of Φ with period Φ_0 . (Here Φ_0 is the quantum flux unit h/e , where h is Planck's constant and e is the charge of an electron.) For the wavefunction to stay coherent around the ring, the electron must be able to circulate around it without being scattered inelastically (that is, without other degrees of freedom becoming excited). Also, the thermal energy must be negligible and the charge carriers must not become localized in some small part of the ring. Weak, purely elastic scattering of the electrons (by defects, for example) is not detrimental to the persistent current.

Although quantum mechanics ensures the survival of the persistent currents, their magnitude depends on the microscopic arrangement of defects in each specific sample. The fundamental harmonic of the oscillatory persistent current corresponds to the Aharonov-Bohm oscillation, characterized by the quantized period Φ_0 . That the conductance of such rings also exhibits such an oscillation is now widely known. The fundamental vanishes when the current is averaged over the different arrangements of defects in an 'ensemble' of systems that are identical on a macroscopic scale (Y.

are identical on a macroscopic scale (Y. Gefen, personal communication). The second harmonic, $\Phi_0/2$, survives, however, through the effect of 'coherent backscattering' (essentially, the interference between the wavefunctions of two electrons traversing the ring along opposite paths). It is the survival of this harmonic in the experiments of Levy *et al.* that causes the magnetic signal to be enhanced rather than averaged away by the large number of rings. It turns out that the condition for survival of the $\Phi_0/2$ component in this case is the use of a canonical ensemble — that is, one in which the number N of particles (in this case, electrons) is fixed^{3,10}. In fact, one may view the periodicity of I for varying Φ as the result of the variation in the highest occupied electronic level, induced by the constancy of N (ref. 11).

In the simplest case, the 'sign' of the averaged currents will correspond to a paramagnetic induced moment — the energy of the electrons decreases with increasing flux (for small fluxes). But if the electrons' orbital motion interacts strongly with their spin, the moment may become diamagnetic (energy increasing with flux). Levy *et al.* think that the observed effect is diamagnetic, but stress that this identification is as yet very tentative.

An alternative interpretation of the persistent currents (ref. 12 and V. Ambegaokar and V. Eckern, personal communication) relies on electron-electron interactions. These may be repulsive (as a consequence of the Coulomb interaction) or attractive (as in the case of the Cooper pairs of conventional superconductivity). These will lead to paramagnetic or diamagnetic moments respectively, and would not be sensitive to spin-orbit coupling. So the 'sign' of the moments (as well as the relevance of the constancy of N) should provide a means of determining which of the two approaches — the independent-electron picture or that which incorporates electron interactions — is the most appropriate.

It would be important to perform these experiments with a single ring, although these are still more difficult. Here,

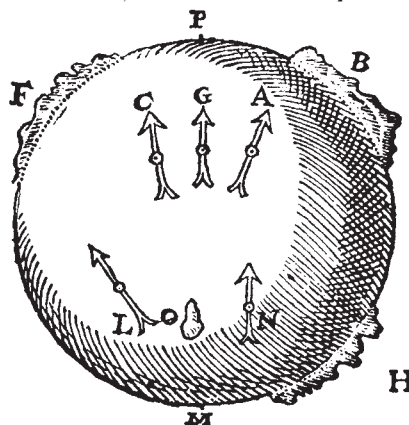
aperiodic fluctuation effects due to the field acting on the edge of the ring would be superimposed on the periodic signal. Such effects have been discovered in preliminary experiments by R. Webb and coworkers at IBM (personal communication). These effects should average out in an ensemble of thin rings; but their importance has yet to be estimated in the experiments described by Levy *et al.*

GEOMAGNETISM

A slow-moving field

Ronald T. Merrill

IN 1600 William Gilbert described the Earth's magnetic field as originating from permanently magnetized material with two magnetic poles — essentially a bar magnet. Today, the Earth's main magnetic field is still described at the Earth's surface as mainly dipolar (about 90 per cent). Its origin, however, is thought to be in the liquid outer core (at a depth of 2,881 to 5,150 km), which is at a temperature



The world of William Gilbert — a terrella (globe-shaped magnet) showing the north-seeking property of magnetic needles. (From *De Magnete*, 1600; Ann Ronan Picture Library.) too high for there to be any permanent magnetization. Instead, it occurs through dynamo action: a weak magnetic field is amplified in the core through the motions of an electrical conducting field. These fluid motions also cause variations in the Earth's magnetic field with periods that range from those as short as can be resolved (about one year) to those that exceed 100 million years. The most dramatic and best documented of these variations are polarity reversals, in which the geomagnetic north and south poles essentially change places over the course of a few thousand years. The average rate of reversals over the past twenty million years is about 4.5 every million years, whereas between 118 and 83 million years ago (the Cretaceous superchron) there were no, or very few, reversals. Such long-term variation appears to be a robust result and is interpreted to reflect changes in the core's boundary conditions². It

Other possible causes of the persistent currents, such as clustering of magnetic impurities or flux-dependent interactions between them, are also yet to be ruled out. □

Yoseph Imry is in the Department of Nuclear Physics, Weizmann Institute of Science, Rehovot 76100, Israel, and the IBM T. J. Watson Research Center, Yorktown Heights, New York 10598, USA.

should be no surprise to learn of additional attempts to discern other geomagnetic phenomena that have long-term variations. Prevot *et al.*³ have now updated the palaeointensity record and conclude that it also shows evidence of long-term variations. If correct, this could have important consequences for the thermal history of the Earth and the origin of the Earth's magnetic field.

Prevot *et al.* are not the first to try to determine the palaeointensity record over geological time. More than twenty years ago, Smith argued that there has been a trend towards an increase in palaeointensity over the past few hundred million years¹. The measurements used by Smith were made before there was good documentation for the Cretaceous superchron and some of the palaeointensity methods used would not be judged acceptable by today's standards. A newer analysis by Pal and Roberts⁵ suggested the intensity of the Earth's magnetic field was greater than usual during the Cretaceous superchron, supporting the prediction of some geomagnetists that a stronger magnetic field gives stability against reversals⁶. But many palaeomagnetists reacted with alarm to the data and methods used by Pal and Roberts to arrive at this conclusion; in particular, the data were sparse and came from rocks (sediments and altered marine igneous rocks) from which it was doubted that reliable intensities could be obtained. Prevot *et al.* seem to be the first to take up the challenge to produce a more credible analysis of the existing data. They have done this by using published data that are restricted to volcanic rocks and that have been obtained by applying a palaeointensity method known as the Thellier method. They have produced what is probably the best estimate to date of the variation of palaeointensity with time for the past 250 million years. Their main finding is that the palaeointensity is significantly lower during the Mesozoic between 125 and 175 million years ago. Although the age limits of this low-intensity interval are not yet well determined, they appear to be discernibly different from those defining the Cretaceous

1. Büttiker, M., Imry, Y. & Landauer, R. *Phys. Lett.* **A96**, 365–368 (1989).
2. Levy, L.P., Dolan, G., Dunsmuir, J. & Bouchiat, H. *Phys. Rev. Lett.* **64**, 2074–2077 (1990).
3. Bouchiat, H. & Montambaux, G. *J. Phys. (Paris)* **50**, 2695–2707 (1989).
4. Cheung, H.-F., Gefen, Y. & Riedel, E.K. *Phys. Rev. Lett.* **62**, 587–590 (1989).
5. Entin-Wohlman, O. & Gefen, Y., (1989) *Europhys. Lett.* **8**, 477–481 (1989).
6. Altshuler, B.L. & Spivak, B.Z. *Sov. Phys. JETP* **65**, 343–347 (1987).
7. Aharonov, Y. & Bohm, D. *Phys. Rev.* **115**, 485 (1959).
8. Byers, N. & Yang, C.N. *Phys. Rev. Lett.* **7**, 46–50 (1961).
9. Block, F. *Phys. Rev.* **B2**, 109–121 (1970).
10. Cheung, H.F., Gefen, Y., Riedel, E.K. & Shih, W.H. *Phys. Rev.* **B37**, 6050–6062 (1988).
11. Imry, Y. *Proc. 1990 Nato ASI on Coherence Effects in Mesoscopic Systems* (ed. Kramer, B.) Les Arcs, France (in the press).
12. Altshuler, B.L., Khmel'nitskii, D.E. & Spivak, B.Z. *Solid St. Commun.* **48**, 10–14 (1983).

superchron. This was not expected and the authors suggest that this will probably lead to changes in models of heat transfer in the Earth's interior.

The above results come from the discipline of palaeomagnetism, a discipline that attempts to determine the primary remanent magnetization of rocks (that is, the magnetization acquired when the rock formed). Such measurements have been shown to yield valuable data on the properties of the Earth's magnetic field⁷. The difficulty of this task varies from one rock to another because of a number of problems, the most serious of which is distinguishing the secondary magnetization — magnetization acquired after the rock formed — from the primary magnetization. It turns out that it is usually far easier to obtain reliable palaeodirection data than palaeointensity data. Nevertheless, it is usually conceded that reliable palaeointensity data can be obtained from some archaeological artefacts and rocks, and that the most reliable method of doing this is the Thellier method. This method, developed by E. and O. Thellier⁸, involves using rocks or archaeological artefacts that have acquired a thermal remanent magnetization, or TRM, during cooling from high temperatures. The TRM is usually found to be linearly proportional to the intensity of the inducing magnetic field. In its initial form, Thellier's method worked when the magnetization was a pure TRM; numerous reheatings and measurements were made to check that this was so. Very few samples yielded reliable intensities; instead, most results were discarded because there was evidence of secondary magnetization or because chemical alteration occurred during the reheating experiments. Subsequently, numerous modifications to Thellier's original method have been made that reduce the number of reheatings involved and sometimes introduce ways to 'correct' for adverse secondary magnetization effects. Although these modifications have made it possible to obtain many more palaeointensity estimates than before, they have also increased the chances for error. Controversy has followed (see refs 9 and 10).

In addition to the controversy over the reliability of the Thellier method and other palaeointensity methods, there is

the problem of how to interpret a 'reliable' palaeointensity estimate derived from a single volcanic unit. If estimates from Thellier's method are correct, then the numerous results obtained from applying this method to samples formed over the past 10,000 years indicate that the field intensity was roughly 40 per cent higher 2,000 years ago and 40 per cent lower 6,000 years ago than it is today⁷. An additional problem is that the Earth's magnetic field is not a perfect dipolar field, but has significant non-dipolar components.

Prevot *et al.*³ are well aware of all these problems. They argue that errors in the method, contributions from the non-dipole field, and short-period variations in the dipole intensity will be averaged out over time. This may well be correct, but are the 87 values used that span about 50 million years sufficient to demonstrate a low magnetic field intensity during the Mesozoic? The conservative scientist will probably dismiss all the Mesozoic inten-

sity estimates as premature. But it is important to recognize that Prevot *et al.* have carried out the most reliable summary to date and at the very least one can draw the important conclusion that there is no convincing case at this time for a high palaeointensity during the Cretaceous superchron. Moreover, given the difficulties in obtaining reasonable palaeointensity estimates and that standard deviation goes as the square root of the number of data used, it seems probable that studies in the near future will also yield a low-intensity estimate for the Mesozoic. Perhaps most certain of all is that the Mesozoic low-intensity estimate provided by Prevot *et al.* will provide considerable fodder for models dealing with the origin of the Earth's magnetic field and evolution of the Earth's interior. □

Ronald T. Merrill is in the Departments of Geophysics and Geological Sciences, University of Washington, Seattle, Washington 98195, USA.

RIBOZYMES

Expanding the RNA repertoire

Geoffrey North

RNA is remarkable for the way it can combine the characteristic ability of DNA to be a template for self-replication with the characteristic ability of proteins to catalyse specific chemical reactions. Until now the catalytic properties of RNA

enzymes, or ribozymes, have been limited to reactions involving phosphodiester bonds, usually of substrates that are also RNA. But, as became clear at a recent meeting*, this may soon change with the development of procedures for the selec-

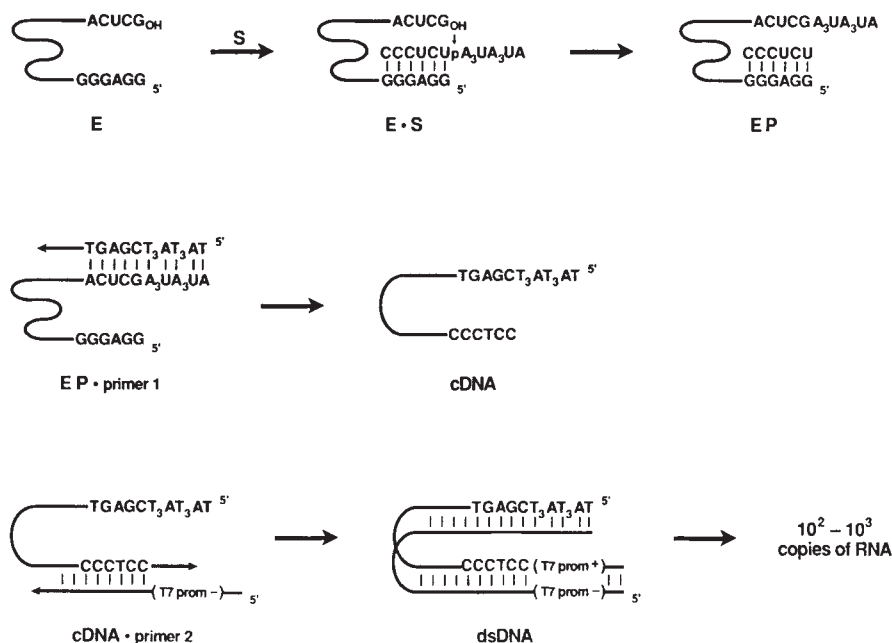


FIG. 1 Scheme of the cycle used by Robertson and Joyce to select RNA enzymes according to ability to cleave oligonucleotide substrates. The ribozyme (E) binds substrate (S) by complementary pairing, and the 3' hydroxyl of the terminal guanosine of the ribozyme attacks the susceptible phosphodiester bond transferring the 3' portion of the substrate to the 3' end of the ribozyme, creating a site for hybridization of an oligodeoxynucleotide primer of cDNA synthesis. After synthesis of the second strand of the cDNA the DNA is transcribed into RNA, and the cycle can begin over again. P, product; ds, double stranded. (From Robertson, D.L. & Joyce, G. *Nature* **344**, 467-468; 1990.)

- Gilbert, W. *De Magnete* (Dover, New York, 1958).
- McFadden, P. & Merrill, R. *J. geophys. Res.* **89**, 3354-3362 (1984).
- Prevot, M., Derder, M., McWilliams, M. & Thompson, J. *Earth planet. Sci. Lett.* **97**, 129-139 (1990).
- Smith, P. *Geophys. J. R. astr. Soc.* **13**, 483-486 (1967).
- Pal, P. & Roberts, P. *Nature* **331**, 702-705 (1988).
- Olson, P. & Hagee, L. *J. geophys. Res.* **95**, 4609-4620 (1990).
- Merrill, R. & McElhinny, M. *The Earth's Magnetic Field* (Academic, London, 1983).
- Thellier, E. & Thellier, O. *Ann. Geophys.* **15**, 285-376 (1959).
- Aitken, M., Allsop, A., Bussell, G. & Winter, M. *Rev. Geophys.* **26**, 3-12 (1988).
- Walton, D. *Rev. Geophys.* **26**, 15-22 (1988).

tive amplification of RNA molecules with new, predetermined activities.

The idea behind attempts to select RNAs with particular properties is simple: in essence, it is the equivalent of evolutionary selection at the level of individual molecules. Thus, starting with a set of variants on the ribozyme derived from the self-splicing intron of *Tetrahymena* precursor ribosomal RNA, Robertson and Joyce have selectively amplified RNAs according to their ability to catalyse the cleavage of a DNA substrate (*Nature* **344**, 467–468; 1990). Robertson and Joyce devised a scheme in which the ability of the catalyst to cleave DNA was linked to copying of the RNA molecule into DNA by reverse transcriptase (see figure). In this way, they selected variants with increased ability to cleave DNA from a set of deletion mutants of the 'wild-type' ribozyme.

A similar approach has been used to explore the sequence requirements for activity at certain positions of the same *Tetrahymena* ribozyme. Rachel Green (Massachusetts General Hospital, Boston) reported how a ligation reaction catalysed by the ribozyme was arranged to create a substrate for reverse transcription into complementary DNA and then amplification by the polymerase chain reaction (PCR). A set of roughly 250,000 variants of the wild-type ribozyme was made at nine sites chosen by phylogenetic sequence comparison. From this set RNAs were selected with catalytic activity at least as great as that of the starting wild-type RNA. The sequences of these RNAs suggest that complex interactions crucial to the function of the ribozyme occur between certain combinations of the varied bases.

To expand the repertoire of reactions that can be catalysed by ribozymes, a good starting point would be a set of RNAs that can selectively bind novel ligands. To obtain such molecules, Andrew Ellington (Massachusetts General Hospital, Boston) has linked the replication of RNA to its ability to bind to an affinity chromatography column. Ellington started by synthesizing random sequence DNA, which is linked to sequences that allow the DNA to be transcribed into RNA, and at a later stage the RNA to be copied back into DNA.

Transcription of the random sequence DNA, of average length 100 bases, gave a yield of RNA expected to contain about 10^{15} different sequences. This RNA was passed over columns containing specific dye molecules. The RNA that bound to the column was retained and copied by reverse transcription into DNA. This was amplified by PCR, and then the whole cycle was repeated. After five rounds, most of the selected RNA had bound to the dye used to select it, and not to the

* The 1990 Cold Spring Harbor RNA processing meeting, 16–20 May.

A long and ancient road

In a two-part report in this month's issue of *Antiquity* (64, 210–220, 1990) Hillam *et al.* announce an exact date for the Neolithic timber pathway known as the Sweet Track. The track, one of the oldest known man-made structures in Europe, was constructed from timber felled in the winter and early spring of 3807–3806 BC. Although the track



had already been dated approximately by radiocarbon, the authors pinned down its precise age by matching tree rings in the preserved timbers to a 'master' 7,000-year chronology of oaks from Irish and German bogs.

In the second part of the report, Coles and Coles discuss the track itself (some of the remains of which can be seen in the picture) and the archaeological context. It consisted of a single line of planks, about 1,800 metres in length, which ran across a swamp in what is now the British county of Somerset. Some of the wood used was felled slightly later than the rest, implying that the track underwent a series of repairs a few years after it was built. From the dating of these repairs, the authors conclude that the track could

have been in use only for about ten years, before being overwhelmed by the swamp.

A range of artefacts and other remains recovered from the peat, either alongside or beneath the track, can therefore be linked with a precise time, allowing us a glimpse of some of the activities of Stone Age men and women and the environment in which they lived. For example, from analysis of beetle remains found near the track it seems that British winters were 2–4 °C colder and summers 2–3 °C warmer than they are today.

Among the artefacts recovered are potsherds, a tomahawk, a comb, toggles, a spoon and even a piece of grass rope. Of particular note are two axeheads preserved in practically mint condition — one made of jadeite, the other of flint. The flint has been identified as coming from the flint mines in Sussex, whereas the jadeite is thought to be from the Swiss Alps. Clearly, some kind of European marketing system was in use even then.

Philippa Lloyd

other, chemically quite similar dyes. Some of the selected RNAs have been sequenced, and RNAs independently selected by a particular dye turn out to have only limited sequence similarity. This may be significant — mutagenesis of two of the selected RNAs showed the sites in the RNAs that are crucial for dye-binding, and it turns out that there is a good correspondence between these sites and sites where the sequences of some of the independently selected RNAs are identical. The implication is that the same structural solution to the problem of binding specific dye molecules has been found more than once in the selection procedure.

Two of the dyes used in these experiments are structurally similar to the bio-

logical cofactor nicotinamide adenine dinucleotide (NAD), and starting from RNAs selected to bind these dyes Ellington has been able to apply the same procedure to select for binding to NAD. He intends to go on and select for RNAs that bind structural analogues of the transition states for particular chemical reactions, in the hope that the RNA will turn out to catalyse the reaction, and in this way to develop novel ribozymes.

An alternative approach to the synthesis of RNA enzymes with new catalytic activities would be that of rational design based on knowledge of the structure of an existing ribozyme. In no case so far is the full three-dimensional structure of a ribozyme known, but A. Pardi (University of Colorado, Boulder) reported progress in

the determination by two-dimensional NMR spectroscopy of the structure of a ribozyme derived from self-cleaving plant viroid RNA; this ribozyme is in the 'hammerhead' class, so-called because of the secondary structure the RNA is predicted to adopt based on a pattern of conserved sequences. Although the NMR data Pardi's group have obtained so far are insufficient for full structure determination, they have already revealed the presence of a stable hairpin-loop.

Much of the interest in the catalytic potential of RNA stems from the wish to test the feasibility of an 'RNA world' early in evolution, in which self-replicating RNA systems existed in the absence of proteins. With the advent of proteins and the genetic code, it is supposed that RNAs retreated from the central functional role in cells, being left with activities such as pre-mRNA splicing and translation which involve dealing with other RNA molecules and to which they are therefore particularly suited.

The observations reported by A. Lambowitz (Ohio State University) provide what may be a glimpse of an interaction between RNA and protein that may have evolved relatively recently. A few years ago Lambowitz's group discovered that the removal by splicing of introns of the group I class from precursor RNAs in *Neurospora* mitochondria depends on a nuclear gene; it turned out that the gene encodes the enzyme mitochondrial tyrosyl transfer RNA synthetase. They have now found that this enzyme binds directly to the 'core' region of introns, containing the conserved sequences that define the group I class of introns, and that it does so by the same binding site by which it binds to tRNA.

Interestingly, the sequences of the intron to which tyrosyl tRNA binds can in principle be folded in the pattern predicted for group I introns from phylogenetic and mutagenesis studies. At the same time, they exhibit some of the characteristics of tyrosine tRNA. At some stage in its past history, random changes to the intron sequence presumably created a variant that sufficiently resembles tyrosine tRNA for it to bind tyrosyl tRNA synthetase, and for some reason this interaction proved beneficial. In the course of subsequent evolution, the intron may have come to depend on this interaction with tyrosyl tRNA synthetase for splicing.

With the intense interest in the potential activities of RNA both for the insights they may give into early events in evolution and the possible benefits of exploiting catalytic RNAs, it seems that the prediction made by Olke Uhlenbeck (University of Colorado, Boulder) that the 1990s will be the 'decade of RNA' may not be wide of the mark. □

Geoffrey North is an assistant editor of *Nature*.

The Sun's disturbing behaviour

Kenneth H. Schatten

THE "unique series of solar cosmic-ray events" reported by Mathews and Venkatesan on page 600 of this issue¹ is one of several indications that, underlying the usual solar cycle (with a period of about 11 years), levels of solar activity have been increasing gradually over the past 400 years. As the Sun has engaged routinely in such increases and decreases in activity (varying on timescales of a few hundred years) for at least the past few thousand million years, this observation is scarcely a cause for alarm. But it does mean that we should expect the complex atmospheric effects that accompany such increased activity.

Increases in solar activity are manifest in a wide range of effects, including

greater numbers of sunspots and solar flares, variations in the solar constant (the amount of solar energy incident on the Earth), greater fluxes of solar ultraviolet and extreme ultraviolet radiation, solar X-rays and protons, and geomagnetic storms in the Earth's atmosphere. We can survive these effects readily, being protected by both the atmosphere and the Earth's magnetic field. Some of our more recent and sensitive creations, however (such as satellites, radio communications and electrical power systems), can suffer from these anomalies.

In particular, these consequences of solar activity can create, in turn, the following terrestrial effects: fade-out of over-the-horizon radio communication

The solution for teething troubles

It is a commonplace in anthropology that the present can provide a key to understanding the past. Writing in the *American Journal of Physical Anthropology*, Brown and Molnar¹ demonstrate the effectiveness of the approach by answering a question that has puzzled those in the field for well over half a century². The question is to do with marks left by some unknown cultural practice that seems to have endured in our hominid ancestors for more than two million years³.

The physical traces of the practice are clear, usually consisting of grooves on interproximal tooth surfaces, at or near the cemento-enamel junction, principally in the molar and premolar regions, and occurring more commonly in males than females. The grooves are horizontal, forming semicircular channels on both of two apposed teeth, with the combined channel ranging in diameter from 1 to 4 mm.

As Paul Bahn⁴ explained in his News and Views article on the topic last year, several proposals have been put forward to account for these observations — root caries (dental erosion, in which bacteria have attacked the tooth surface); gritty saliva having been propelled through the teeth; and fibre processing resulting from the stripping of highly abrasive grasses and leaves. Perhaps the most common suggestion has been that the grooves were caused by use of a probe of bone or wood — a toothpick of sorts — to palliate dental problems such as gingival irritation. But all of these hypotheses remained at the level of conjecture in the absence of systematic observations on living humans still following practices that might have produced the grooves.

Brown and Molnar have now made such observations — to my mind the answer is in, and it is simple and satisfying. Analysing both the dentition of aboriginal skulls from South Australia and cinematographic records of aboriginal society⁵, they show that the lesions result from the stripping of animal sinews. The preparation and use of animal sinews has been recorded on two films, *The Woomera* and *Aboriginal Spears*. Each shows the stripping of kangaroo sinew through clenched posterior teeth, producing a thin but strong cord for fixing a carved wooden peg to a woomera (spear-thrower) or a carved wooden tip to a spear shaft. Thinned and pliable sinews, like dental floss, slip between the contact points of adjacent teeth and reach the cemento-enamel junction. Continual stripping in the same region produces abrasion at the junction and, in due course, interproximal grooving.

Because such lesions are very similar in all populations in which they have been reported, it is reasonable to infer that they have a common cause and it would be difficult to find one more fitting than that identified by Brown and Molnar. Palaeo-anthropology is replete with unsolved problems — it is good to see even one removed from the roster.

Robert B. Eckhardt

Robert B. Eckhardt is in the Department of Anthropology, 409 Carpenter Building, Pennsylvania State University, University Park, Pennsylvania 16802, USA.

1. Brown, T. & Molnar, S. *Am. J. phys. Anth.* **81**, 545–553 (1990).
2. Siffre, A. *Bull. Soc. Prehist. fr.* **8**, 741–743 (1911).
3. Boaz, N.T. & Howell, F.C. *Am. J. phys. Anth.* **46**,

93–108 (1977).

4. Bahn, P.G. *Nature* **337**, 693 (1989).

5. Richardson, N. *Australian Aboriginal Studies* **88**, 56–58 (1988).

and other radio variations, enhanced satellite drag and early re-entry, anomalies in or damage to satellite electrical systems, satellite radiation degradation including possible communications problems, induced anomalous voltages in electrical power systems and long-line networks, blackout of high frequency polar communications, errors in VLF (very low frequency) navigation systems, and possible exposure to hazardous radiation (in rare events) from within high-altitude aircraft and spacecraft.

During the present solar cycle, there has already been an electrical blackout in Quebec, Canada, despite the use of more resistive circuit breakers to protect electrical networks from the power surges induced by geomagnetic storms. High-latitude electrical systems are most at risk: during the 1958 solar maximum, for example, Sweden lost its electrical grid for several days. Another startling manifestation of the current solar cycle was the geomagnetic storm of March 1989, which was among the three most severe in recorded history. At this time, there was a dramatic increase in the number of 'lost objects' in near-Earth orbit (including small parts from old satellites, boosters, explosive bolts and so on).

Other possible effects of solar variations upon the Earth are less certain, but may be real. There is reasonably good, albeit circumstantial, evidence that the Earth's climate responds to solar activity. For example, during the seventeenth century, a lull in solar activity was accompanied by a cooling of the Earth's climate, represented by the Little Ice Age over Europe. Conversely, the Earth may experience warming when the Sun is more active, as recent ACRIM (Active Cavity Radiometer) and ERB (Earth Radiation Budget) experiments show². Greenhouse gases are, however, likely to be a more potent warming influence. A number of Sun-weather correlations have been reported^{3,4}; but these tend to be statistical in nature, lacking a physical mechanism, and they often disappear as new data are obtained.

The cosmic-ray events reported by Mathews and Venkatesan are referred to as ground-level enhancements or events (GLEs), because the influence of the solar flares extends all the way to the ground, and is in fact detected by ground-based neutron monitors; normally, cosmic-ray variations are restricted to the upper levels of the atmosphere. Only particles of very high energy (about a billion electron volts) will reach the ground, because of interactions with the Earth's magnetosphere and atmosphere. Furthermore, the usual effect of solar flares is simply to reduce the cosmic-ray flux of galactic origin (a so-called Forbush decrease), by a process in which the solar plasma and magnetic field from the flare

sweeps interplanetary space clear of these energetic galactic particles. The hot flare gases also carry a large number of very-high-energy particles from the Sun, but these are usually insufficient in number and energy to offset the interplanetary sweeping effect. In the case of a GLE, however, they are able both to offset the Forbush decrease and to cause an increase in ground-level energetic particle fluxes. Many flares in the current solar cycle have produced particles with sufficient energies and in sufficient numbers to cause GLEs⁵. G. Heckman (personal communication) has reported that the solar proton flux has been generally higher during this cycle than during others, which is consistent with the large number of GLEs reported.

Despite these reports of a high level of solar cosmic rays, other measures of solar activity (such as sunspot numbers and radio fluxes) seem likely to place the present cycle as only the second largest in the past 400 years. Already the cycle's activity seems to be waning relative to that in 1989. But solar cycles are notorious for their unpredictable behaviour. Although

as their average period is 11 years, it can vary from 8 to 17 years, and amplitude variations can occur on virtually every timescale, which makes predictions by Fourier analyses as unreliable as they are in weather forecasting. So whereas we can forecast solar cycles on decadal timescales using the 'dynamo' or 'precursor' methods (see refs 6 and 7), we have yet to understand fully the intricacies of the solar cycles' aberrant behaviour. The surprisingly large solar proton fluxes and their attendant GLE, for example, were not predicted. □

Kenneth H. Schatten is in the Goddard Space Flight Center, Greenbelt, Maryland 20771, USA.

1. Mathews, T. & Venkatesan, D. *Nature* **345**, 600–602 (1990).
2. Willson, R. C. & Hudson, H. S. *Nature* **332**, 810–812 (1988).
3. Van Loon, H. & Labitzke, K. *J. Clim.* **1**, 905 (1988).
4. Wilcox, J. M., Scherrer, P. H., Svalgaard, L., Roberts, W. O. & Olson, R. H. *Science* **199**, 185 (1973).
5. Bieber, J. W., Evenson, P. & Pomerantz, M. A. *Eos* (in the press).
6. Brown, G.M. in *Solar-Terrestrial Predictions Proc.* (eds Simon, P.A., Heckman, G. & Shea, M.A.) **1** (National Oceanographic and Aeronautics Association, Boulder, Colorado, 1986).
7. Schatten, K.H. & Sofia, S. *Geophys. Res. Lett.* **14**, 632–635 (1987).

PALAEOMAGNETISM

A polished view of remagnetization

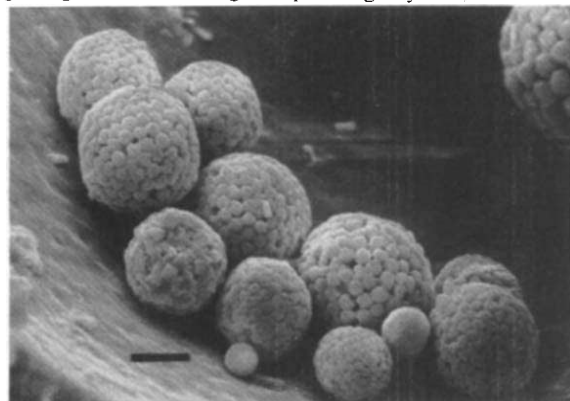
Richard L. Reynolds

MAGNETISM in rocks has provided a traditional tool for studies of the Earth's ancient geomagnetic field. These studies have tended to rely on the assumption that the direction of magnetization was 'frozen in' during formation of the rock. But many sedimentary rocks formed during the Palaeozoic acquired their remanent magnetization through alteration processes that occurred after deposition of the sediment. The causes and geological significance of this phenomenon have been much debated. On page 611 of this issue, Suk *et al.*¹ show that remagnetization can result from chemical transformations induced by crustal fluids. Their results imply that palaeomagnetism can provide information on the composition of these fluids and the way that their flow is controlled by geological processes such as mountain building.

Some carbonate sediments deposited in the oceans of the early and middle Palaeozoic, about 530 to 320 million years ago, are now exposed in the Appalachian mountains of North America as limestone and dolomite, and also farther inland in areas less disturbed by tectonic activity. These rocks provide palaeomagnetic data

that have for many years been used to reconstruct the behaviour of the North American crustal plate. The remanent magnetization of these sedimentary rocks is due to the iron oxide magnetite² (Fe₃O₄). The common occurrence of magnetite, derived from older rocks and soils, in other modern and ancient sediments contributed to the confidence placed in the results from these limestones.

During the past eight years, however,



Scanning electron micrograph of pyrite framboids identified from marine sediments by Morse and Cornwell¹⁷. Suk *et al.*¹ show that such pyrite can be transformed to magnetite in ancient limestone. Scale bar, 3 µm.

evidence has accumulated that the remanent magnetization of many carbonate sediments was not acquired at the time of deposition, thereby invalidating some previous interpretations of the palaeo-

magnetic data. Instead, magnetization seems to have been acquired over a limited time span during the late Palaeozoic, from about 310 to 250 million years ago²⁻⁴.

How can magnetite be responsible for this magnetic overprint? Two explanations have emerged. First, the magnetite may have formed chemically after deposition either by conversion from pyrite⁵ (FeS₂) or by new growth perhaps related to alteration of clay⁶ or to microbial degradation of hydrocarbons²⁻⁷. Sparse magnetite concentrated from remagnetized carbonate rocks and examined under the scanning electron microscope has a grain shape and a surface texture that suggests a post-depositional, chemical origin⁸. Often, the magnetite particles are spherical (typically 3 to 40 micrometres in diameter), resembling framboidal pyrite (see figure). The second possibility is that remagnetization results from a reorientation of the magnetism in magnetite — regardless of its origin — following its exposure to moderately high temperatures⁹.

The careful analytical work by Suk *et al.*¹, using scanning transmission electron microscopy and electron diffraction, helps to resolve these uncertainties. They first identified magnetite in thin and polished slabs of the carbonates, and examined its spatial relationship to other minerals in the rock. In addition, polishing and milling enabled the authors to see inside individual crystals of magnetite (most of which are less than one micrometre across) and thus to discover that some crystals had centres of pyrite and margins of magnetite. This, and the observation that the aggregates of magnetite crystals have a shape identical to that of framboidal pyrite, indicates that magnetite has formed from pyrite and not the other way around.

The approach of cutting and polishing specimens for examination under reflected-light and electron microscopes can have enormous advantages. Other palaeomagnetic studies would benefit from such an approach, because internal textures and compositional differences within magnetic grains can reveal their origins and alteration histories.

Suk *et al.* point out that the direction of remanent magnetization in remagnetized rock probably records the direction of the Earth's magnetic field while the magnetite formed from pyrite. How other factors, such as thermal effects^{4,10} and tectonic strain¹¹, may have influenced the remagnetization process requires further examination; but the chemical basis for these studies is now clear.

Alteration of pyrite to magnetite has occasionally been found in pyritic shales that have been heated locally by nearby igneous intrusions over a geologically short duration and at temperatures probably greater than 200 °C; but it has

not been reported previously for widespread alterations that occur over millions of years and at temperatures mainly less than 200 °C. In previous magnetic studies of sedimentary rocks, pyrite has been considered important only in respect of its tendency to replace depositional magnetite^{12,13}, a process that can diminish the strength of magnetic properties but does not affect their orientation.

The observations of Suk *et al.* have implications for large-scale geological processes. The late Palaeozoic remagnetization event has been ascribed to chemical alteration caused by warm, saline fluids that were expelled from sedimentary rocks squeezed near zones of collision between crustal plates during building of the Appalachians¹⁴. Other manifestations of such an event in mid-continent North America include the distributions and patterns of hydrocarbon and metal deposits, as well as post-depositional growth of minerals other than magnetite, such as potassium feldspars. The new results add further support to the idea of a link between remagnetization of limestones and chemically active fluids, whatever their origin^{15,16}. In these rocks at temperatures generally less than 200 °C, heat alone could not have converted pyrite to magnetite: oxidizing agents in fluids must have also played a part. The extent of the alteration would have been controlled by the types and concentrations of these agents, by temperature¹⁰, by the focusing of pathways of fluid flow^{2,6,16}, and by the presence and concentration of pre-existing pyrite. All of which suggests that remagnetization of rocks will become viewed not as inconvenient but as serendipitous in providing a record of thermochemical alteration related to the fluid-flow history of sedimentary basins. □

Richard L. Reynolds is with the US Geological Survey, Box 25046, MS 964, Denver, Colorado 80225, USA.

1. Suk, D., Peacor, D.R. & Van der Voo, R. *Nature* **345**, 611–613 (1990).
2. McCabe, C., & Elmore, D.R. *Rev. Geophys.* **27**(4), 471–494 (1989).
3. Van der Voo, R. *Geol. Soc. Am. Mem.* **172**, 447–470 (1989).
4. Miller, J.D. & Kent, D.V. *Geology* **16**, 588–591 (1988).
5. McCabe, C., Van der Voo, R., Peacor, D.R., Scotese, C.R. & Freeman, R. *Geology* **11**, 221–223 (1983).
6. McCabe, C., Jackson, M. & Saffer, B. *J. Geophys. Res.* **94**, 10429–10443 (1989).
7. Elmore, R.D. *et al. Nature* **325**, 428–430 (1987).
8. McCabe, C., Sassen, R. & Saffer, B. *Geology* **15**, 7–10 (1987).
9. Kent, D.V. *Geophys. Res. Lett.* **12**, 805–808 (1985).
10. Jackson, M., McCabe, C., Ballard, M.M. & Van der Voo, R. *Geology* **16**, 592–595 (1988).
11. Craddock, J.P. & van der Pluijm, B.A. *Geology* **17**, 416–419 (1989).
12. Karlin, R. & Levi, S. *J. Geophys. Res.* **90**, 10373–10392 (1985).
13. Canfield, D.E. & Berner, R.A. *Geochim. cosmochim. Acta* **51**, 645–660 (1987).
14. Oliver, J. *Geology* **14**, 99–102 (1986).
15. Bethke, C.M., Harrison, W.J., Upson, C. & Altaner, S.P. *Science* **239**, 261–267 (1988).
16. Clendenin, C.W. & Duane, M.J. *Geology* **18**, 116–119 (1990).
17. Morse, J.W. & Cornwell, J.C. *Mar. Chem.* **22**, 55–69 (1987).

The midnight Sun

THE Earth's rotation provides us with light in intermittent doses. Daylight is amply bright, often inconveniently so; but at night even the best lighting provides only a feeble substitute. The obvious solution, says Daedalus, is to store samples of daylight for release at night.

A primitive form of this technology already exists. Luminous materials such as doped zinc sulphide can be 'charged' with light, which excites certain energy states in the crystal lattice: as these re-emit the stored energy, the material glows feebly in the dark for a few hours afterwards. Light recovery, however, is dismal: a mere few parts per million. Furthermore, the emitted light has only one colour, typically a depressing green.

But Daedalus is undaunted. He points out that photographic emulsions, thanks to close attention to crystal form, doping and the cunning use of sensitizing dyes, can attain wide-spectrum photon-capture efficiencies of several per cent, while luminous re-emission can be very efficient indeed (as in fluorescent lamps). So DREADCO's physicists are hard at work on the design of a truly advanced solid-state light-storage medium, or 'Lightstorm'.

For a balanced, white emission, Lightstorm will need three separate excited states of different energies, most easily obtained by mixing three different phosphors. To avoid wasteful internal scattering, they will be suspended in a medium of the same refractive index. A Lightstorm coating will appear clear but darkened by its light absorption, like transparent 'smoked plastic' resin. A storage capacity of around 50 joules per gram should enable a Lightstorm surface to charge up totally during the day and to glow cheerfully all night with a brightness of about one per cent of that of natural daylight. Existing artificial lighting does no better — our eyes adjust wonderfully to alterations of brightness. The even, non-dazzling glow of a Lightstorm ceiling should be very easy on the eyes. It would, of course, slowly fade as the night wore on, but then so does human activity.

Lightstorm will revolutionize the illumination industry. As wallpaper and ceiling paint it will light our houses at night; on the roads it will provide automatic free street lighting, road markings and illuminated signs. Nocturnal crime will plummet, while the poster and neon-sign industries will plumb new depths of garish vulgarity. All sorts of objects, from telephones and pens to socks and bicycles, will be made luminous for easy identification in the dark. The 24-hour Lightstorm greenhouse will boost horticultural productivity. And the saving of electricity will wonderfully curtail carbon dioxide production.

David Jones

Antibodies against AIDS proteins

SIR—The human immunodeficiency virus HIV-1 encodes several regulatory proteins (in addition to the virion structural proteins *gag*, *pol* and *env*), including *vif*, *vpr*, *vpu*, *tat*, *rev* and *nef*¹. We have investigated the presence of antibodies against some of these regulatory proteins in human sera to determine whether any of them could indicate the progression of HIV infection from the asymptomatic stage to full-blown AIDS.

It is known that putative *vif* protein, expressed in bacteria, reacts with sera from HIV-1-infected individuals at all clinical stages². By contrast, antibodies against a transregulatory gene product, *rev*, of HIV-1 predominantly occur in the sera of patients with ARC (AIDS-related complexes)³. We have re-examined the reactivity of antibodies against *vif* and *rev* in sera of HIV-1-infected individuals at various clinical stages and, in contrast to previous reports³, our findings are consistent with the idea that antibodies against *vif* protein appear early in HIV-1 infection and disappear as clinical signs of AIDS appear, whereas antibodies against *rev* protein are present at all clinical stages of HIV-1 infection.

The *vif* gene encodes a protein of relative molecular mass 27,000 (27K) and of unknown function; mutants of HIV-1 deleted in the *vif* gene produce approximately 1,000-fold less infectious virus compared with the wild type⁴. The *vif* protein seems to be necessary for the HIV-1 to recognize CD4-positive lympho-

cytes as the *vif* deletion mutants could still spread HIV-1 from cell to cell by fusion as efficiently as the wild-type virus. This suggests that *vif* is a minor structural component of HIV-1. Functional expression of the HIV-1 *rev* protein is required for synthesis of *gag* and *env*^{5,6}: *rev* is a 20K nuclear regulatory phosphoprotein⁷ which

Prevalence of antibodies against *vif* and *rev* in HIV-1-infected individuals

Disease stage	No. of Total <i>vif</i> Ab ⁺ /tested (%)	No. of Total <i>rev</i> Ab ⁺ /tested (%)
Asymptomatic (CDC group I)	17/52 (32.6)	18/52 (34.6)
Asymptomatic (CDC group II)	8/22 (36.3)	9/22 (40.9)
ARC patients (CDC group III)	0/18 (0)	4/18 (22.2)
AIDS patients	0/27 (0)	6/27 (22.2)

HIV-1 positive sera were determined by western blots and gp41-recombinant ELISA. Ab, antibody.

induces structural gene expression by activating the sequence-specific nuclear export of incompletely spliced messenger RNA species of HIV-1 (ref. 8). This protein increases stability of unspliced viral mRNA but does not affect the stability of multiple spliced viral mRNAs not containing the *rev*-responsive element⁹.

We have analysed antibodies against *vif* and *rev* in several HIV-1-infected individuals using the *vif* and *rev* proteins expressed in SF9 cells infected with recombinant baculoviruses carrying either the full length *vif* or *rev* gene of HIV-1 under the control of the polyhedrin gene promoter. The level of intracellular *vif* and *rev* expression in the infected cells 72 h after infection was approximately 25 and 15 per cent, respectively of the total cellular protein (see figure). *vif* and *rev* could both react with antibodies in certain sera.

We next examined sera from 52 HIV-1-infected healthy donors, 22 HIV-1-infected healthy donors at risk, 18 ARC patients and 27 AIDS patients (see table). Our results are consistent with the suggestion that antibody against *vif* is produced early in HIV-1 infection and disappears as the disease progresses to ARC and AIDS. This disappearance could signal the appearance of the clinical signs of HIV-1 infection. In contrast, antibody against *rev* is present at all stages of HIV-1 infection.

These results differ from those of Chandra *et al.*³, who found the *rev* antibody predominantly in the sera of ARC patients³. This difference could be due to the source of *vif* and *rev*. Chandra *et al.*³ used antigens expressed in bacteria which, in our hands, show a high degree of non-specific cross-reactivity whereas *vif* and *rev* expressed in insect cells show a clean background in western blots. We also note that human antibodies against *vif*

and *rev* are relatively weak compared with other HIV-1 proteins, indicating that in natural infection these proteins are poor antigens. Furthermore, antibodies produced in rabbits against *vif* proteins expressed in insect cells demonstrate that *vif* is poorly immunogenic.

YAIR DEVASH

KEVIN REAGAN

DAVID WOOD

Medical Products Department,
E. I. DuPont Co.,
Wilmington, Delaware 19898, USA

JOHN TURNER

Department of Medicine,
Graduate Hospital, One Graduate Plaza,
Philadelphia, Pennsylvania 19146, USA

MARK PARRINGTON

C. YONG KANG*

Department of Microbiology and
Immunology,
University of Ottawa,
Ottawa, Ontario K1H 8M5, Canada

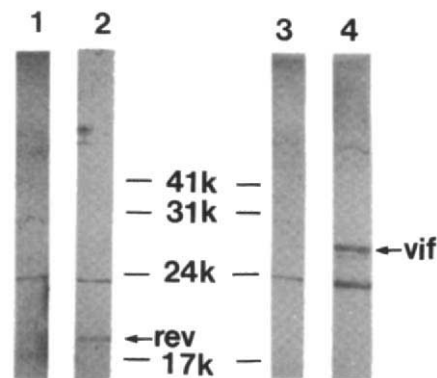
* To whom correspondence should be addressed.

1. Gallo, R. *et al.* *Nature* **333**, 504 (1988).
2. Kan, N.C. *et al.* *Science* **231**, 1553–1555 (1986).
3. Chanda, P.K., Ghayeb, J. & Wong-Staal, F. *AIDS Research and Human Retroviruses* **4**, 11–16 (1988).
4. Strebel, K. *et al.* *Nature* **328**, 728–730 (1987).
5. Feinberg, M.B., Jarrett, R.F., Aldovini, A., Gallo, R.C. & Wong-Staal, F. *Cell* **46**, 807–817 (1986).
6. Sodroski, J. *et al.* *Nature* **321**, 412–417 (1986).
7. Cochrane, A., Kramer, R., Ruben, S., Levine, J. & Rosen, C.A. *Virology* **171**, 264–266 (1989).
8. Malim, M.H., Hauber, J., Le, S.-Y., Maizel, J.V. & Cullen, B.R. *Nature* **338**, 254–257 (1989).
9. Felber, B.K., Hadzopoulou-Cladaras, M., Cladaras, C., Copeland, T. & Pavlakis, G.N. *Proc. natn. Acad. Sci. U.S.A.* **86**, 1495–1499 (1989).

Trans-kingdom promiscuity

SIR—Two examples of horizontal transfer of DNA from a prokaryote to eukaryote have so far been described. The best-studied case is the transfer of agrobacterial T-DNA from the bacterium to the genome of a susceptible plant cell¹. This conjugation-like exchange introduces plant oncogenes and facilitates a complex symbiotic relationship between these two organisms. A second, unexpected case of horizontal gene transfer has recently been demonstrated in the laboratory between *Escherichia coli* and the budding yeast *Saccharomyces cerevisiae*². The biological relevance of this strange union is still unclear, but the mechanism of transfer clearly resembles that of bacterial conjugation. Intrigued by the idea of "generic trans-kingdom sex", as discussed in News and Views³, we investigated whether the fission yeast *Schizosaccharomyces pombe* can also act as the recipient in bacterial conjugation.

Our assay for DNA transfer was based on the two-plasmid conjugation system reported previously². Briefly, one large bacterial plasmid serves as a 'helper' to mobilize in *trans* a second plasmid containing the ColE1 origin of transfer (*oriT*). Evidence for transfer of the *E. coli*



Western blot of *vif* and *rev*. Recombinant baculovirus carrying either *vif* (AcNPV-*vif*) or *rev* gene (AcNPV-*rev*) was used to infect *Spodoptera frugiperda* (SF9) cells. The infected cells were collected 72 h post infection, total cellular protein was subjected to SDS-PAGE, transferred to nitrocellulose filter and incubated with HIV-1-infected human sera. The reaction was completed after washing and incubations with horseradish peroxidase-conjugated anti-species antibody and precipitable substrate (4-chloro-1-naphthol). Lanes 1 and 2, wild-type baculovirus (AcNPV)-infected SF9 cell extracts; lane 2, AcNPV-*vif*-infected SF9 cell extracts; lane 4, AcNPV-*vif*-infected SF9 cell extracts. The band just below the 24K marker is a non-specific protein.

based plasmid YE13 to *S. pombe* was obtained by mixing bacteria and yeast and selecting Leu⁺ yeast prototrophs. The results of a typical experiment are shown in the figure. We observed production of Leu⁺ *S. pombe* at frequencies comparable to those reported using *S. cerevisiae* as the recipient². The absolute requirement for the helper plasmid, which provides essential mobilization and transfer functions for *E. coli*/*E. coli* conjugation, strongly suggests that a conjugal mechanism is used in the *E. coli*/*S. pombe* DNA transfer. Furthermore, a transformation mechanism is unlikely as exogenous DNA is completely ineffective under these conditions.

Analysis of five independent Leu⁺ yeast 'transconjugants' yielded results expected of *S. pombe* strains harbouring an episome. All five isolates grew slowly under selection and exhibited mitotic instability for the Leu⁺ phenotype such that on average only 22 per cent of cells in such a culture retested as Leu⁺. In addition, Southern blot analysis of genomic DNA showed that all had inherited YE13 DNA sequences: three carried intact

YE13 and two carried plasmids that had undergone rearrangements (data not shown). The level of mitotic instability and the frequency of rearrangements are both typical of YE13 plasmids introduced into *S. pombe* by transformation¹.

The finding that DNA can be transferred directly from *E. coli* to *S. pombe* is significant for several reasons. Fission-yeast geneticists may find applications for this 'conjugation' method as an alternative to DNA-mediated transformation for introducing cloned genes into *S. pombe*. DNA manipulated on most pBR322 derivatives will be suitable for conjugation-mediated transfer since pBR322 carries a functional *ori-T*. Furthermore, given the great evolutionary distance between budding and fission yeasts, one may be encouraged to test other eukaryotes as potential recipients in the laboratory. The prevalence and significance of trans-kingdom conjugation in nature remains to be determined. In this regard, we note that unexpectedly significant similarities have recently been

reported between a yeast and a bacterial alcohol dehydrogenase³, between a retroviral and a bacterial RNase H (ref. 6), and between fungal and bacterial isopenicillin N synthetases⁷. Could sexually promiscuous bacteria have grafted these unusual limbs onto the evolutionary tree?

ROBERT S. SIKORSKI

WILLIAM MICHAUD

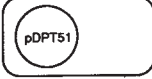
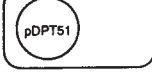
HENRY L. LEVIN

JEFF D. BOEKE

PHILIP HIETER

Department of Molecular Biology and Genetics,
The Johns Hopkins University School of Medicine,
Baltimore, Maryland 21205, USA

1. Zambryski, P., Tempe, J. & Schell, J. *Cell* **56**, 193–201 (1989).
2. Heinemann, J. & Sprague, G.F. *Nature* **340**, 205 (1989).
3. Stachel, S.E. & Zambryski, P.C. *Nature* **340**, 190 (1989).
4. Heyer, W.-D., Sipiczki, M. & Kohli, J. *Molec. cell. Biol.* **6**, 80–88 (1986).
5. Williamson, V.M. & Paquin, C.E. *Molec. gen. Genet.* **209**, 374–381 (1987).
6. Doolittle, R.F., Feng, D.-F., Johnson, M.S. & McClure, M.A. *Q. Rev. Biol.* **64**, 1–30 (1989).
7. Ramon, D., Carramolino, L., Patino, C., Sanchez, F. & Penalva, M.A. *Gene* **57**, 171–181 (1987).

<i>E. coli</i> Donor	Leu ⁺ <i>S. pombe</i>	Transfer efficiency
	692	5 × 10 ⁻⁶
	0	<7 × 10 ⁻⁹
	0	<7 × 10 ⁻⁹
	0	<7 × 10 ⁻⁹
No bacteria	0	<7 × 10 ⁻⁹

Transfer of plasmid-encoded information from *E. coli* to *S. pombe*. The *E. coli* strain SB21 (ref. 2) containing the broad-host-range 'helper' plasmid pDPT51 or a transformant of this strain which also contained YE13 were mixed with a leu1 *S. pombe* strain and plated directly on yeast medium lacking leucine. YE13 contains a functional *ori-T*, replicates autonomously in *S. pombe* and contains the *S. cerevisiae* LEU2 gene, which complements the *S. pombe* leu1 mutation. Requirements for the transfer process were investigated by manipulating the *E. coli* genotype (shown schematically) or by providing exogenous YE13 DNA. Transfer efficiency is the number of Leu⁺ colonies per potential yeast recipient.

METHODS. *S. pombe* strain Sp659 (h⁺h⁺ leu1-32/leu1-32 ura4/ura4 ade6/ade6) and the various *E. coli* donor strains were grown separately to saturation in YE (0.5 per cent yeast extract) and LB (plus antibiotics), respectively. The antibiotics trimethoprim (200 µg ml⁻¹) for pDPT51 or tetracycline (12.5 µg ml⁻¹) for YE13 were added to LB as required for plasmid selection. Cultures were washed once and resuspended in TNB (50 mM Tris, pH 7.6, 0.05 per cent NaCl). Routine transfer experiments were performed by mixing 200 µl 5 × concentrated Sp659 (~10⁸ cells) with 200 µl 10 × concentrated bacteria (~10⁹ cells). The mixtures were immediately pelleted, resuspended in 200 µl TNB and plated to SD medium. To provide exogenous YE13, 10 µg CsCl-purified plasmid DNA was added directly to TNB and plated with the cells.

Capable biocatalysts

SIR—In their recent review on asymmetric chemical synthesis¹, Brown and Davies commented that "... the methods of biotechnology are most suited to the production of natural molecules or closely related homochiral compounds and ... by contrast potentially all homochiral compounds are accessible ... by chemical asymmetric synthesis". We believe this conclusion undermines the already demonstrated capabilities of biotransformations.

A few examples of the preparation of homochiral compounds by biotransformations that are either operating on the multitonne scale now, or are sufficiently highly developed processes awaiting commercial-scale operation, should set the record straight. All the processes described below produce compounds of high optical purity, greater than or equal to 95 per cent enantiomeric excess.

- (1) (S)-Naproxen ((S)-6-methoxy- α -methyl-2-naphthaleneacetic acid) by esterase resolution of racemic Naproxen (International Bio-Synthetics, BV-IBIS).
- (2) L-2-chloropropionic acid by halo-hydrolysis treatment of the racemic chloro acid (ICI).
- (3) (R)-(-)-2,2-dimethyl-1,3-dioxolane-4-methanol by bio-enantioselective oxidation of the racemic compound (IBIS).
- (4) (R)-Glycidyl butyrate by lipase resolution of the racemic ester (Genzyme).
- (5) (S)-Atenolol ((S)-(-)-4-(2-hydroxy-3-isopropylaminopropoxy)-phenylacetamide) via enantioselective bio-epoxidation of the prochiral methyl-4-(2-propenyloxy)-phenylacetate (IBIS).

These selected examples, together with the increasing number of enantioselective biotransformations being discovered in

an extremely broad range of organic chemical structural types, clearly show the versatility of asymmetric synthesis using enzymes or whole-cell systems. We believe that asymmetric synthesis using natural catalysts is a highly attractive proposition for the preparation of novel, optically pure organic compounds. Reviews and textbooks (refs 2–5, for example) are available to encourage organic chemists to measure the attributes of these methods against the use of man-made asymmetric catalysts and reagents.

R. J. PRYCE

Shell Research Ltd,
Sittingbourne, Kent ME9 8AG, UK

S. M. ROBERTS

Department of Chemistry,
University of Exeter, Devon EX4 4QD, UK

BROWN AND DAVIES REPLY—The needs of both the chemical and pharmaceutical industries for pure organic compounds with a high specificity of action will ensure rapidly expanding application of asymmetric synthesis over the next several years. In our article¹ we endeavoured to stress the approaches devised by synthetic organic chemists. We drew a distinction between the use of homochiral catalysts and reagents, as defined, and more classical approaches, including resolution of racemates and use of the 'chiral pool'. We touched on the alternative possibility of using the methods of biotechnology, acknowledging the 'spectacular selectivity' of enzymes. We stressed a potential advantage of the chemical approach in that the range of asymmetric transformations available through organic chemistry is far wider than the range available

through enzymology, as nature conducts reactions through a relatively restricted set of procedures.

The examples quoted by Pryce and Roberts permit us to make direct comparison of the 'state of the art' in the respective areas. Indeed, four of their five flagships for biotransformation are resolutions of racemic compounds by enzymes and not asymmetric syntheses! The fifth is an epoxidation (enzyme catalyst not specified) affording the β -blocker (*S*)-atenolol, carried out by the Shell subsidiary IBIS. By comparison, chemical asymmetric epoxidation is being carried out on a multi-ton scale by the Sharpless titanium-tartrate method (see p.632 of our review) to produce related glycidols. Other examples of asymmetric synthesis used on a large scale in industry are current; we cite the rhodium catalyst-based isomerization which is the key step in the Takasago process for the synthesis of the ubiquitous flavour ingredient menthol, competing

with a cheap and abundant natural source. Consequently, we stand by our views expressed in our article, which we believe to be fair and accurate.

There is ample scope for both chemical and biochemical methods in asymmetric synthesis. To some extent these will be provided by healthy competition which will sharpen the development of new science. We welcome the opportunity to participate in this competition with our friends in the biotechnology community.

JOHN M. BROWN
STEPHEN G. DAVIES

*Dyson Perrins Laboratory,
Oxford University, South Parks Road,
Oxford OX1 3QY, UK*

1. Brown, J.M. & Davies, S.G. *Nature* **342**, 631 (1989).
2. Jones, J.B. *Tetrahedron* **42**, 3351-3403 (1986).
3. *Enzymes in Organic Synthesis* Ciba Fdn Symp. **111** (1985).
4. Whitesides, G.M. & Wong, C.H. *Angew. Chem. int. Ed. Engl.* **24**, 617-638 (1985).
5. Davies, H.G., Green, R.H., Kelly, D.R. & Roberts, S.M. *Biotransformations in Preparative Organic Chemistry* (Academic, London, 1989).

Toxic oil disaster

SIR—The events that took place in the Madrid area of Spain in 1981 probably represent the worst recorded outbreak of food-borne chemical intoxication ever documented. Between May 1981 and March 1983, 340 deaths and more than 20,000 cases were registered¹. Subsequent research on the causes of the outbreak,

however, has been singularly unrewarding².

Epidemiological evidence has implicated the consumption of aniline-degraded, and subsequently reprocessed, rapeseed oil as the causative agent, but attempts to reproduce the chemical composition of suspect samples of oil have been unsuccessful. Furthermore, the pathology seen in the affected patients was previously unknown and no comparable animal model response has been demonstrated.

We report here progress in addressing these two fundamental inhibitions to successful examination of the mechanisms of this syndrome: the production of oil of comparable chemical composition to case-related oils; and the development of a discriminating *in vitro* bioassay. Our results show that the necessary tools for the mechanistic study of the Spanish toxic oil syndrome are available. Further work now has a reasonable prospect of resolving this eight-year-old mystery.

Samples of crude rapeseed oil, including one of the *Jet Neuf* variety believed to have been used to produce the original toxic oil, were adulterated with aniline, stored and then processed under conditions mimicking as closely as possible the conditions that pertained in the 1981 incident. Figure 1 shows HPLC traces of these adulterated oils and of similarly processed control oil not denatured with aniline. For comparison, case-related and control oils of

known provenance (supplied by the *Fondo de Investigaciones Sanitarias de la Seguridad Social*) are also shown. Several compounds are present in the aniline-treated oils, and radiochemical studies show that these are aniline-derived.

In vitro toxicological studies using mouse neuroblastoma and rat liver epithelial cells successfully discriminated between aniline-adulterated oils and controls, and some HPLC fractions from each, when the test material was presented to the cells in a predigested form. Without this pretreatment, nonspecific toxic effects were seen in all cases. Figure 2 shows examples of the effects of exposing cells to extracts of control and aniline-treated oil samples.

P. T. SLACK
G. M. WOOD
C. B. MALLINSON
P. N. GILLATT
J. B. ROSSELL
W. J. REID
D. F. LEWIS
R. C. COTTRELL

*Leatherhead Food Research Association,
Randalls Road,
Leatherhead, Surrey KT22 7RY, UK*

1. *World Health Organisation Toxic Oil Syndrome Mass Food Poisoning in Spain* (WHO Regional Office for Europe, Copenhagen, 1984).
2. *Fondo de Investigaciones Sanitarias Toxic Oil Syndrome's Reference* (Archive Ministerio de Sanidad y Consumo, Spain, 1990).

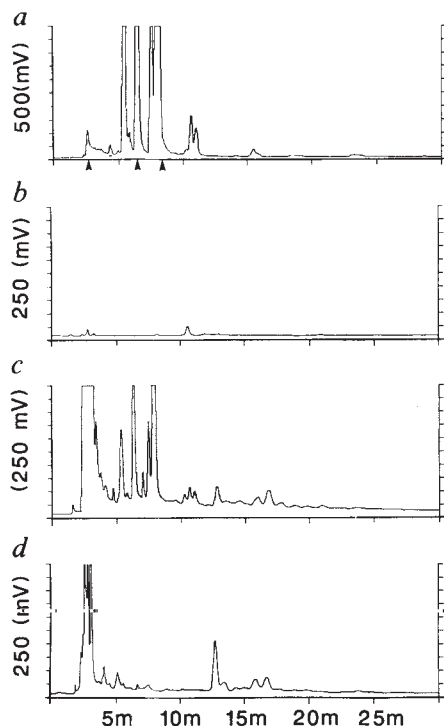


FIG. 1 HPLC traces of *a*, ³H-aniline-adulterated and then processed French rapeseed oil, compared with: *b*, its appropriate processed control; *c*, genuine case related oil KM489; and *d*, an appropriate control of similar age. Column C18 ODS, solvent methanol:water 95:5 by volume. Monitor absorbance at 254 nm. Main radioactive peaks marked.

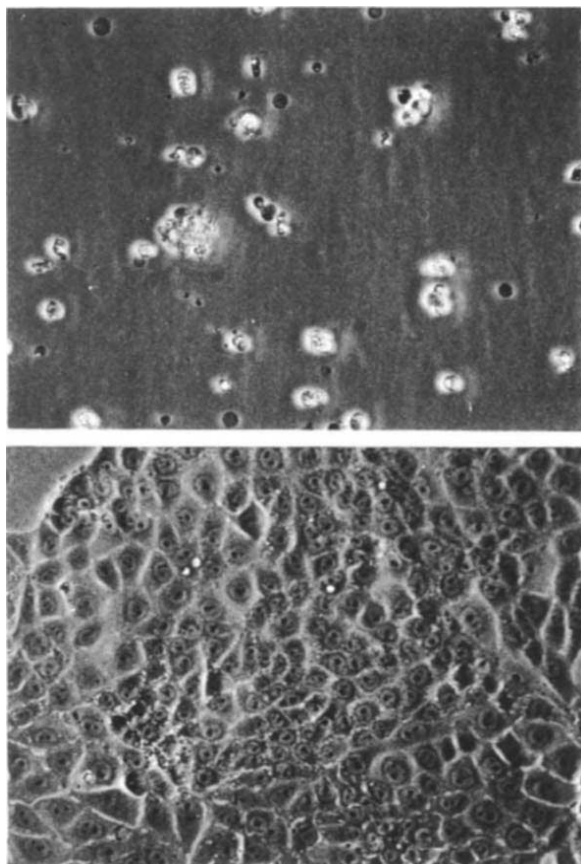


FIG. 2 Effect of extracts from aniline-treated rapeseed oil (top) and an appropriate control oil (bottom) on rat liver epithelial cells. Magnification, $\times 300$.

Direct repeats

SIR—The identification of a retinoic-acid response element by de Thé *et al.*¹ led to the interesting observation that this element contains a perfect direct repeat of the sequence motif GTTCAC. We wish to point out that the thyroid hormone response element (T3RE) from the rat growth-hormone promoter²⁻⁴ contains two copies of an imperfect direct repeat of the motif AGGTAA. Mutation of this sequence to a perfect direct repeat leads to a substantial increase in T3 responsiveness²⁻⁴. Furthermore, we have shown³ that this perfect direct repeat by itself is a functional T3RE. The rat growth hormone T3RE also contains a third imperfect copy of the hexamer inverted relative to the other two^{4,5}. Making this sequence a better match to the T3RE consensus sequence AGGT(C/A)A also increases T3 response⁴; it has also been shown that a perfect inverted repeat of this consensus sequence by itself functions as a strong T3RE (ref. 5). Remarkably, a functional element conferring response to T3 (or retinoic acid) can be constructed from two copies of a hexameric recognition site arranged as either direct or inverted repeats.

Although the retinoic-acid receptor can induce transcription of rat growth hormone, it is clear the proposed consensus hexameric T3 response motif is only distantly related to the proposed retinoic-acid receptor motif GTTCAC. Thus, it would be interesting to know if the T3 receptor can bind to and induce expression via this latter sequence. Further, how can a single protein bind to sequence elements arranged as either direct or inverted repeats? How is the function of hormone response elements affected by the number and orientation of such repeats? Do response elements that contain multiple copies of such repeats, such as those found in rat growth hormone, osteocalcin and laminin, bind mixed oligomers of different receptor monomers?

RONALD J. KOENIG

Division of Endocrinology,
University of Michigan Medical Center,
Ann Arbor,
Michigan 49109, USA

GREGORY A. BRENT
P. REED LARSEN*

Thyroid Unit and
Howard Hughes Medical Institute*,
Department of Medicine,
Brigham and Women's Hospital,
Boston, Massachusetts 02115, USA

DAVID D. MOORE
Department of Molecular Biology,
Massachusetts General Hospital,
Boston, Massachusetts 02114, USA

1. de Thé, H. *et al.* *Nature* **343**, 177–180 (1990).
2. Koenig, R. J. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **85**, 5031–5035 (1988).
3. Brent, G. A. *et al.* *J. biol. Chem.* **264**, 178–182 (1989).
4. Brent, G. A. *et al.* *Molec. Endocrin.* **3**, 1996–2004 (1989).
5. Glass, C. K. *et al.* *Cell* **54**, 313–323 (1988).

Lipid test

ALTHOUGH the irradiation of foodstuffs has been approved in many countries, as an important method for preventing food spoilage and foodborne diseases, worldwide acceptance and trade in irradiated foods have been hindered by the lack of an appropriate means of control¹. Although several methods for the identification of irradiated foods have been proposed, radiation-induced changes are generally very small. The most easily measured changes are those produced by chain reactions, whereby the initial chemical change is multiplied many times. We have accordingly explored the potential of the radiation-induced oxidation of lipids with the formation of hydroperoxides as a suitable chain reaction for identification.

We propose the use of lipid hydroperoxides (LOOH) as indicators of the irradiation of egg powder, which is a potential vector of salmonellosis, whose low bulk and high monetary value provide the necessary economic and technical incentives for radiation processing, and which contains high and approximately equal proportions of proteins and lipids (see figure).

Several batches of commercially available spray-dried eggs obtained from two manufacturers were irradiated by gamma-rays at a dose rate of about 14 kGy h⁻¹ under three distinct sets of conditions: in open, sealed and evacuated polyethylene pouches, respectively. After irradiation, lipid was extracted by a chloroform:methanol mixture (2:1 v/v) and the amount of hydroperoxides determined directly by a modified ferrous thiocyanate method².

The formation of hydroperoxides appears to depend only on the radiation dose. There is at first an exponential increase of hydroperoxide with dose, followed, beyond about 4 kGy, by a linear increase at least with samples to which the atmosphere has access. The linear regions of these curves, when extrapolated backwards, intercept the dose axis at about 2 kGy, which is interpreted as the dose

required to overcome natural radioprotective capacity. With samples sealed from the atmosphere, on the other hand, hydroperoxide formation is found to saturate at about 3 kGy, but the hydroperoxide concentration (about 1.5 mmol LOOH per kilogram of lipid) is more than ten times higher than in unirradiated samples.

We have also investigated the stability of radiation-induced hydroperoxides over a period of more than 6 months. After an initially rapid decay for about 10 days, the first-order decay of LOOH proceeded for about three half-life periods (see figure). The rate of the first-order decay was approximately proportional to the dose received by the sample. Thereafter, decay continued more slowly; the level of LOOH remaining 6 months after irradiation was still higher than the background value of unirradiated samples (0.11 ± 0.07 mmol kg⁻¹).

We have shown that reduction factors for *Salmonella* of between 10³ and 10⁵ can be achieved by irradiating dehydrated eggs up to 3 kGy in the presence of air, and up to 5 kGy in the absence of air, without adversely affecting the organoleptic properties^{3,4}. At these dose levels, the irradiated samples can be unambiguously identified by the elevated hydroperoxide levels at least 6 months after the treatment. Hot storage, the only alternative method for elimination of *Salmonella* from egg powder (1 to 2 weeks at 49–55 °C)⁵ does not generate hydroperoxides significantly above the background level. On the other hand, boiling irradiated samples in water for several minutes does not destroy radiation-induced hydroperoxides.

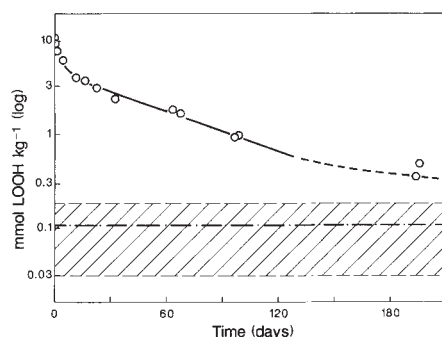
The advantage of the proposed method is that the measured parameter is inherent to the food itself. Other dehydrated foods rich in lipids and with an extensive inner surface, such as dehydrated milk and soya flour, exhibit similar behaviour. But before a method can be developed to measure the irradiation absorbed by a material, further work is necessary to characterize the effects of environment on the response and its time-dependence.

BRANKA KATUŠIN-RAŽEM

BRANKA MIHALJEVIĆ

DUŠAN RAŽEM

Ruder Bošković Institute,
PO Box 1010
41001 Zagreb, Yugoslavia



Time-dependence of radiation-induced lipid hydroperoxides LOOH in whole egg powder irradiated with 4 kGy in equilibrium with air. Shaded area, background level of LOOH.

1. Delincee, H., Ehlermann, D.A.E. & Bogl, K.W. in *Health Impact, Identification and Dosimetry of Irradiated Food* (eds Bogl, K.W., Regulla, D.F. & Suess, M.J.) 58–127 (Institut für Strahlenhygiene des Bundesgesundheitsamtes, Munich, 1988).
2. Gray, J.I. *J. Am. Oil Chem. Soc.* **55**, 539–546 (1978).
3. Matic, S., Mihoković, V., Katušin-Ražem, B. & Ražem, D. *J. Food Prot.* (in press).
4. Katušin-Ražem, B., Ražem, D., Matic, S., Mihoković, V., Kostromin-Sooš, N. & Milanović, N. *J. Food Prot.* **52**, 781–786 (1989).
5. International Commission on Microbiological Specifications for Foods in *Microbiology of Foods Vol. II* (eds Silliker, J.H. *et al.*) 521–566 (Academic, New York, 1980).

Behind the veil

John Cairns

The Cancer Industry: Unraveling the Politics. By Ralph W. Moss. Paragon House: 1990. Pp. 502. \$21.95.

TEN years ago, Ralph Moss wrote a savage denouncement of the treatment of cancer patients and the way the "war against cancer" was being organized. The book was called *The Cancer Syndrome*, and it contained hardly a good word for any of the established forms of treatment or for any of the major figures in the field. The only people to escape condemnation were the proponents of those drugs such as laetrile which, rightly or wrongly, had been judged to be ineffective and were not licensed by the US Food and Drug Administration.

Moss is now offering the same attack, bringing it up to date by the inclusion of more details about the costs of treatment. This is a worthwhile addition. Moss produces some powerful numbers: even in the early 1970s, the average medical costs incurred by cancer patients in New York greatly exceeded the total annual income of the average New York family. (As one in three of the population eventually gets cancer, this means that most families were, at some time, having to devote a year's income to pay for the treatment of cancer — an extraordinary state of affairs, when one realizes that this payment was producing hardly any change in the age-standardized death rate from cancer.)

Since the 1970s, the bill has increased. Last year, it was probably more than \$50 thousand million. As Moss says, "cancer care is not a charity; it is a business — big business", and he reminds us that "someone is receiving much of the money that the cancer victim disburses". The average doctor in the United States would probably say that this is as good a thing to spend your money on as anything else and that, anyway, much of the money is being siphoned off by the lawyers. I myself believe that families (and nations) should put education of the young at the top of the list and high-tech treatment for the old almost at the bottom (especially if it does not work), and I suspect that this is most people's opinion. For example, Surgeon-General Koop was quoted as saying, at the time of his retirement, that he had "brought a generation of Americans to realize that the promotion of good health and the prevention of disease is largely within their grasp, and that by changing lifestyles they can accomplish more than by worrying about high-tech medicines." I wish that Moss had extended this section of his book and considered what he believes we should be spending our money on.

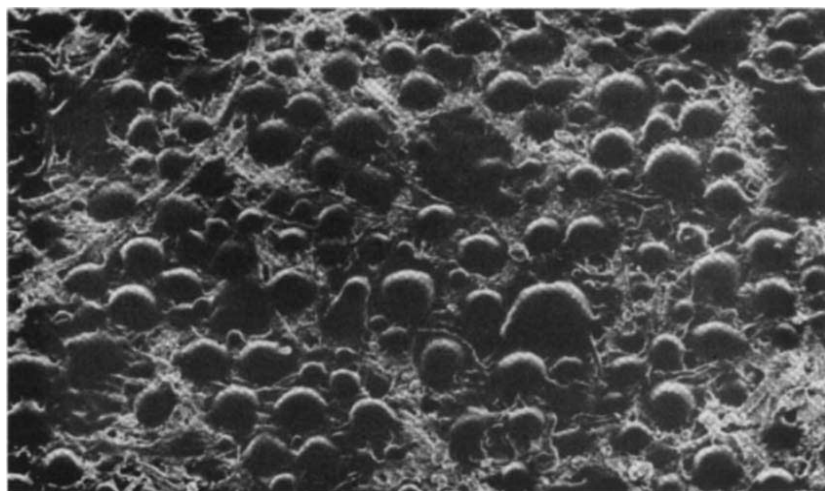
When Moss discusses different forms of treatment for cancer, his relentless negativity clouds his judgement. He is sarcastic on the subject of five-year survival rates, mainly because he does not really understand competing risks and the problem of determining who is cured and who is not. He has many bad things to say about surgery, radiation and chemotherapy. Although some of his criticisms are surely justified, I think he is too gloomy. Who knows, one day he himself may develop cancer of the prostate and be only too thankful for the palliative action of diethylstilbestrol (something he does not mention in his book, even though this is a well-established treatment for a common form of cancer). The subject of mammography provides a good example of Moss's propensity to look on the worst side of things. It is now abundantly clear that screening by mammography can reduce subsequent mortality from breast cancer; this was shown in a New York trial and in the subsequent experience of the Scandinavian countries (which, conveniently for everyone else, differed greatly in their rates of screening). Certainly, there have been arguments as to whether the whole exercise is cost-effective and whether the benefits are likely to outweigh the risks, but these are separate, subsidiary issues. Typically, Moss elaborates the risks but does not at any point mention any of the

evidence of benefit.

It was not obvious from *The Cancer Syndrome* why Moss should be so angry. In his new book, he has put into the preface an important piece of personal history. He describes how, in the late 1970s, he was fired from his job as assistant director of public affairs at the Memorial Sloan-Kettering Cancer Center because he thought that their trials of laetrile had been rigged to give a negative result. This explains the theme in most of the chapters, which is that there is probably some wondrous cure for cancer out there that is being suppressed by the chemical industry and the cancer establishment. I can see the attraction in believing that somewhere in the world around us there lurks the answer to our every need — a love potion, a recipe for transmuting base metals into gold, even an instant cure for cancer. But the evidence is not good. After all, if something like laetrile really worked (in the sense that penicillin worked, when first tested) it is hardly conceivable that the many people who have been given laetrile would keep silent about their miraculous cure.

Despite its obvious defects, the book contains many good things, and I wish it could be made required reading for those who are high up in the US cancer establishment, because it would give them at least an inkling of the public's mounting displeasure. As the contractors for the Department of Defense are coming to realize, you cannot hope to continue indefinitely to be paid huge sums of money for weapons that do not work. □

John Cairns is in the Department of Cancer Biology at the Harvard School of Public Health, 665 Huntington Avenue, Boston, Massachusetts 02115, USA.



Each of these tiny resin spheres is filled with glue and has been incorporated into the surface of a sheet of paper. When the sheet is pressed onto a firm surface, it becomes stuck as some of the spheres burst, releasing their adhesive. The process can be repeated many times as only a few spheres burst on each occasion. This micrograph of a 'Post It' sticker is reproduced from the new paperback edition of *Under the Microscope: A Hidden World Revealed* by J. Burgess, M. Marten and R. Taylor. Published by Cambridge University Press, on 12 July, £12.95, \$19.95.

Shift in theories

Anthony Hallam

Drifting Continents and Colliding Paradigms: Perspectives on the Geoscience Revolution. By John A Stewart. *Indiana University Press: 1990. Pp. 272. \$35.*

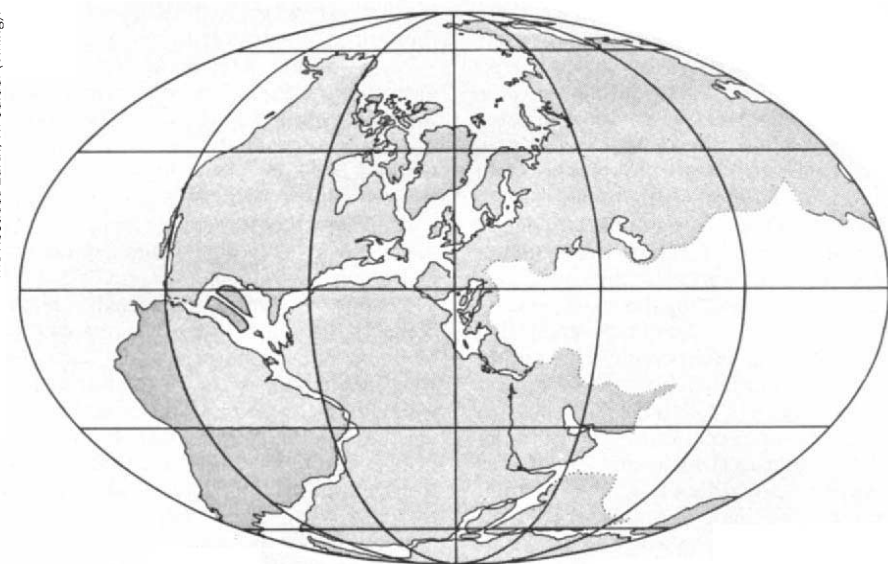
IN RECENT years there has been a huge growth of interest and activity in what one might call the science of science. The more philosophical approach, associated particularly with the names of Popper and Lakatos, emphasizes the intellectual aspects of scientific research, with atten-

science, and offers promising material for the sociologist. More than half of Stewart's book is devoted to a recital of this history, effectively starting with Wegener's theory of continental drift and its generally adverse reception, and continuing in more detail with the new research in geophysics and oceanography that led to the revolution. This is all well-trodden ground, dealt with in numerous books of varying degrees of accessibility to the general public, but Stewart's account is balanced, thorough and well-illustrated, and indicates a good grasp of the subject for someone who is not a geoscientist by training. Full use is made of quotations from some of the leading protagonists,

very similar title of *Drifting Continents and Shifting Theories* (Cambridge University Press). Unlike Stewart, Le Grand is hostile to 'number-crunching' quantitative analysis, and follows Laudan rather than Kuhn in arguing that the fundamental aim of science is to maximize the scope and number of solved empirical and conceptual problems while minimizing the number of anomalies and conceptual problems generated. There is competition between rival research programmes and, within them, competition between theories. This greater emphasis on rational factors does not invalidate Stewart's more sociological approach but is in effect complementary to it. Stewart makes no extravagant claims for the statistical analysis he promotes, and is content to express the modest hope that it may point the way to more illumination in the future, in a variety of subject areas. He should find the current controversy about mass extinctions exceedingly promising material because of the wide variety of 'interest' groups involved, and the fact that nearly all the leading players are alive. Most important, perhaps, no consensus has yet been achieved on the cause of mass extinctions a decade after Alvarez put forward his impact hypothesis. This is in sharp contrast to the speed with which a consensus was reached on the essential correctness of plate tectonics.

One doesn't like to quibble, but it is embarrassing to see Merton's Matthew principle repeatedly referred to as the Matthews principle — Stewart is evidently unfamiliar with the New Testament. Although it is amusing to have a random scientist cited as she rather than he to provide a mild shock for male chauvinists, it is tedious to repeat this throughout the book, especially as Richard Dawkins made the point rather more deftly some years ago. □

Anthony Hallam is Lapworth Professor of Geology, University of Birmingham, Birmingham B15 2TT, UK.



Pangaea — the continents fitted together along the edges of the present continental shelves.

tion being paid to logical analysis, rational deductions and the objective testing of hypothesis by observation or experiment. In contrast to this is the sociological approach initiated by Merton and Kuhn, in which attention is concentrated on the cultural traditions and social structure of the scientific community. Stress is laid on the crucial nature of decisions, such as what will be accepted by the consensus as valid evidence, and the inability of logical rules to specify how these decisions should be made. Among sociologists of science one can distinguish so-called functionalists, who attach greater importance to rational criteria, and constructivists, who focus attention more on the nature of the research process to see how norms and conventions become established. Greater emphasis is laid by the latter group on social factors such as scientists' reputations, style of presentation and institutional prestige. Both groups acknowledge the great importance of individual scientists' need for recognition, leading to much conflict and competition.

The geoscience revolution of the late 1960s, leading to the general acceptance of plate tectonics, is one of the best-documented fields in the history of

to indicate how they evaluated the significance of what they were doing at the time. All well and good so far, except that I was not struck by any new insights.

In the more original part of the book, Stewart develops a model of the decisions needed to generate citation of a paper. He then uses this model to develop quantitative procedures for studying 'global' aspects of this decision process. The quantitative section is rather technical and probably of most interest to US sociologists, but the principal conclusion, which I find reassuring, is that papers are cited on cognitive rather than social grounds. The new paradigm of plate tectonics was accepted more rapidly by those with the most professional interest in it, namely geophysicists seeking some form of global theory. Others, such as structural geologists working in given regions of the continents, were less easily convinced, and subsequent 'negotiations' were required between the two groups, leading to a general acceptance of the need to allow, for example, internal deformation of plates and the existence of exotic terranes.

It is interesting to compare Stewart's book with the recently published volume on the same subject by Le Grand, with the

This year *Nature's* annual new journals review supplement will appear in the issue of 11 October. Publishers and learned societies are invited to submit journals for review, taking note of the following criteria:

- Journals which first appeared after June 1988, and which issued at least four separate numbers by the end of April 1990, will be considered for review. The deadline for submission is the end of June.
- Journals covering any aspect of science are eligible, although those dealing with clinical medicine, engineering and pure mathematics are excluded, as are publications of abstracts.
- Frequency of publication must be at least three times a year.
- The main language used must be English.

When submitting journals for review, please send at least four different issues (the first, the most recent and any two others) of each title to: Book Review Editor, *Nature*, 4 Little Essex Street, London WC2R 3LF, UK. □

The mild-mannered Asiatic Black bear (*Selenarctos thibetanus*) is commonly seen in coniferous and broad-leaved mixed forests, tropical rain-forests and in the Himalayas of China. Many other animals and plants are covered in *The Natural History of China* by Z. Ji, Z. Guangmei, W. Huadong and X. Jialin. The volume illustrates the variety and beauty of China's natural history through the lavish use of colour photographs accompanying the text. Published by Collins, price £14.95.



Phylogeny of a pincushion

James W. Valentine

Invertebrate Relationships: Patterns in Animal Evolution. By Pat Willmer. Cambridge University Press: 1990. Pp. 400. Hbk £32, \$49.50; pbk £12.95, \$22.95.

THE millions of living species of animals can be organized within a taxonomic hierarchy into about 35 phyla, each with distinctive body-plan features. At low hierarchical levels the relations among lineages are often fairly clear, but at increasingly higher levels, the taxa are separated by increasingly large morphological gaps, and their interrelations become correspondingly more speculative. The fossil record is of some help in closing those gaps, but the same principle applies, the relations among higher levels generally being the more enigmatic. Opinions among even the most masterful comparative morphologists have varied bewilderingly; the available evidence has not been a convincing guide to phylogenetic relationships at the scale of phyla, and quite a variety of competing hypotheses have been entertained.

Although phylogenies of the animal kingdom are given a chapter in textbooks, no book-length examination of the problems has appeared since Clark's admirable *Dynamics in Metazoan Evolution* in 1964. Now Willmer, in *Invertebrate Relationships*, provides a much-needed treatment that builds on that foundation, reviewing subsequent work and surveying the entire range of living phyla. It is a splendid book. In the early chapters, Willmer presents an overview of the previous hypotheses and recounts the chief sources of evidence for

the phylogenetic schemes — comparative gross morphology, the fossil record, chemistry and genetics, embryology and ultrastructural studies. She then provides a review, phylum by phylum, of the principal ideas concerning their origins and relationships viewed in the light of that evidence. The accounts of supporting or contravening data are clear, documented by a well-chosen bibliography that brings together a widely scattered literature from many fields. In such a lengthy treatment the reader has the opportunity to return again and again from individual cases to the grand scheme of metazoan radiation, and thus to evaluate thoroughly the main schools of thought about the historical sequences and significance of such body-plan features as the haemocoel, coelomic architectures and segmentation. Willmer's general conclusion is that these features are polyphyletic, and that the living bilaterian phyla, sometimes in groups of two to five but usually singly, can be traced back into a plexus of flatworm-like acoelomates. Non-bilaterians are traced back individually into planula-grade ancestors. The flatworm grade has arisen polyphyletically from the planulas, and the planulas polyphyletically from protozoans. Phylogenetic schemes that depict bilaterian phylogeny as a branching pattern based on primitive trimeric body plans (archicoelomates), or that depict the

protostomes as primitive coelenterates or as primitive bilaterians, are rejected. The diagram illustrating the phylogenetic conclusions contains an empty box labelled "acoelomate platyhelminthes" from which 19 independent lineages proceed (3 of them questioned), looking like nothing so much as a pincushion. As to how the branching proceeded within the box to produce these 19 stocks — there are a few clues in the last chapter but no strong hypotheses. Such a pattern of independent origination of bilaterian lineages requires much convergent evolution, for which a principal support is found in the work of Manton (summarized in *The Arthropoda*, 1977).

It happens that I have been, for several years, writing a similar book that emphasizes evidence from the early fossil record, so perhaps it is unfair of me to say that I found the treatment of the fossils to be the least satisfactory part of this work. Willmer has missed the chance to support many of her contentions much more strongly, particularly her basic viewpoint that convergence abounds and that many architectural components are polyphyletic. On the other hand, she has not destroyed my own venture — there is still something left to be said. Indeed, now we have an up-to-date summing-up that presents, with greater clarity than any other of which I am aware, the implications and potential flaws in many of the competing phylogenetic schemes. In Willmer's defence, the fossil literature is fairly new and (as in every other literature) scattered.

Also fairly new are the exciting molecular trees of animal phyla that have appeared too late for inclusion in this book. These trees are not inconsistent with an early branching of the ancestors of many living phyla among flatworm-grade organisms, and thus they may help to fill in the flatworm 'box' with details of the branching patterns. They also suggest, as Willmer gracefully concedes, that some of her placements of phyla will inevitably require changes (probably first among the annelids and lophophorates). The molecular work is still in a formative stage, however, and it is perilous to say much more at this stage.

Anyone with an interest in phylogeny at high taxonomic levels should own this book, and this applies to undergraduates in biological or palaeontological sciences as well as to advanced students and veteran professionals. It is an excellent sorting-out of a traditionally difficult area. We may be on the verge of a new level of understanding about the relationships among phyla, and this book is all the more valuable for that. □

James W. Valentine is in the Department of Geological Sciences, University of California, Santa Barbara, California 93106, USA.

AIDS TARGETED INFORMATION/ATIN

TARGETS YOUR LITERATURE SEARCH

Expand your base of knowledge about AIDS/HIV with *AIDS Targeted Information/ATIN*. Every month, *ATIN* not only provides abstracts but also in-depth evaluations of the current published scientific literature on AIDS. Written by clinicians and researchers for clinicians and researchers, *ATIN* provides an authoritative command of the world's literature on AIDS. Published by Williams & Wilkins. Indexed. 12 issues per year. \$125.

To order call

1-800-638-6423

Sponsored by the American Foundation for AIDS Research (AmFAR).

Reader Service No. 430

Copies of articles from this publication are now available from the UMI Article Clearinghouse.

Yes! I would like to know more about UMI Article Clearinghouse. I am interested in electronic ordering through the following system(s):

- ☐ DIALOG/Dialorder ☐ ITT Dialcom
☐ OnType ☐ OCLC ILL Subsystem

☐ Other (please specify) _____

☐ I am interested in sending my order by mail.

☐ Please send me your current catalog and user instructions for the system(s) I checked above.

Name _____

Title _____

Institution/Company _____

Department _____

Address _____

City _____ State _____ Zip _____

Phone (____) _____

**UMI Article
Clearinghouse**

Mail to: University Microfilms International
300 North Zeeb Road, Box 91 Ann Arbor, MI 48106

Beating a path

Peter Satir

Ciliary and Flagellar Membranes. Edited by Robert A. Bloodgood. Plenum: 1990. Pp. 431. £63.75, \$102.

IN THESE days of megabuck cell and molecular biology, where delimited areas of science teem with data-hungry laboratories pursuing directed goals, it is unusual to discover an area of some depth and practical importance where 'small' science still prevails. This collection of articles, centred around the properties of ciliary and flagellar membranes of organisms ranging from protists to man, reveals both the advantages and disadvantages of research on problems where the whole world contains the same number of investigators as one article sometimes does in more popular fields. A chief advantage is an individualistic focus on unsolved issues whose solution could yield completely new insights into how cells work, but a corresponding disadvantage is the relatively slow resolution of issues and limited advance per unit time.

By contrast to the axoneme (the internal cytoskeleton of the cilium), which is the subject of several books and of an elegant opening review here by Whitman, the ciliary membrane is not well understood. This is, as Bloodgood points out, the first volume to attempt to review what is known about this membrane in any comprehensive way. Although some of the articles are flawed — being either too narrow or, conversely, overly broad — the book is worth reading from cover to cover. An array of unsolved important problems jumps from virtually every chapter. Here is a membrane domain at once responsible for the initiation of mating in some organisms and unexplained gliding movements in others; a domain that has affinity for special long stringy mastigemes and scales; that interacts with the egg cumulus during ovum transport in mammals or specifically with bacteria in the aetiology of human whooping cough; and a membrane that might even cause sandflies to bite more frequently during the transmission of *Leishmania*. The molecular and mechanistic bases of these phenomena are understood in only a rudimentary way. Yet, as the chapters by Vickerman and Tetley and by Tuomanen in particular show, it might be advantageous to study these properties more closely for clinical purposes.

One question that this diversity of function raises is whether the ciliary membrane is distinguishable from the cell membrane, where diversity would perhaps be more expected. The answer, one of the few on which all the contributors seem to agree, is that the ciliary

membrane differs significantly in composition and properties from the somatic membrane of the cell body. Ciliary and cell membranes are often readily separable because of procedures that induce a clean deciliation in cells, and the authors of several articles discuss results showing that ciliary membranes contain more sterols and are probably stiffer than cell membranes.

In many instances, the ciliary membrane develops above a ciliary necklace, which may be a barrier to free diffusion from cell to cilium in the plane of the membrane and which has glycoconjugates on its surface. The ciliary membrane may grow and is sometimes shed continuously. The outer segment of the vertebrate rod, discussed here by Besharse and Horst, is a spectacular example of continuous growth and differentiation of a specialized ciliary membrane. A promising approach is to use the connecting cilium of the rod to develop antibodies against the necklace and to use these to define some common biochemical features, and eventually functional properties, of the necklace region of all the ciliary membranes.

One wonders whether cyclic nucleotide-gated channels of the rod or of olfactory ciliary membranes (not reviewed in the volume) are present in the less specialized ciliary membranes. Unfortunately, discussion of the channel properties of ciliary membranes is one of the weaker parts of the collection. Another weakness is the coverage of sperm, which is largely restricted to the sperm of mammals and mainly presents information relating to the whole cell rather than to the flagellar membrane. On the other hand, a contribution capturing the essence of the subject — and which points out our current limitations of knowledge — is that by Williams on *Tetrahymena* ciliary membranes.

If you are working in a crowded field of cell or molecular biology and want to switch to one where most of the important questions are still to be answered, one where the subject will tickle your brain, this is a book for you. □

Peter Satir is in the Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York 10461, USA.

Corrections

■ In M. Irving's review of "Molecular Mechanisms in Muscle Contraction" by J. M. Squire (*Nature* **345**, 398; 31 May 1990), the last word of a sentence in the penultimate paragraph was inadvertently omitted. The correct sentence should read: "These are discussed in detail in a lucid and thoughtful chapter by Brenner, who also reviews the relationship between solution studies of the actin-myosin ATPase and muscle fibre mechanics."

■ On page 395 of the same issue, two sentences were transposed out of sequence in J. S. Jones's review of "The Search for Eve" by M. H. Brown. The two sentences at the bottom of the middle column of the review should be in the right-hand column, immediately under the illustration. □

Ocean response to greenhouse warming

Uwe Mikolajewicz, Benjamin D. Santer & Ernst Maier-Reimer

Max-Planck Institut für Meteorologie, Bundesstrasse 55, D-2000 Hamburg 13, FRG

Changes in surface air temperature resulting from a doubling in atmospheric carbon dioxide drive changes in ocean circulation. Results from an ocean general circulation model project a global mean sea level rise from thermal expansion alone to be 19 cm in 50 years. Regional values, however, can vary: a rise of 40 cm is projected in the North Atlantic (owing to reduction of deep-water formation), whereas the level of the Ross Sea actually falls through changes in ocean circulation.

AN increase in sea level is likely to be an important consequence of global warming. Even moderate changes in sea level (less than 50 cm) can have large social and economic effects, particularly in heavily populated regions of the world that lie close to sea level. Projecting regional changes in sea level is therefore extremely important.

Several recent studies have considered the transient response of coupled ocean-atmosphere models to time-dependent CO_2 and greenhouse-gas forcing¹⁻³, but have not discussed sea level changes. Virtually all studies that have examined the sea level changes have used simple one-dimensional pure diffusive or upwelling-diffusive models^{4,5} in which only thermal expansion is treated. These investigations cannot simulate the regional distribution of sea level change. The only study in which this

has been attempted, which included both thermal expansion and extremely simplified ocean dynamics, yielded spatially homogenous sea level changes⁶. Here we consider both thermal effects and the full dynamic effects associated with changes in oceanic general circulation.

We isolate the purely oceanic response to a 'pseudo-transient' atmospheric forcing by monthly mean anomalies in the surface air temperature ($2 \times \text{CO}_2 - 1 \times \text{CO}_2$) derived from atmospheric general circulation model (AGCM) equilibrium-response experiments. We examine the robustness of certain features of the coupled models' oceanic response (such as inter-hemispheric asymmetry in the sea surface temperature (SST) changes) in a simpler uncoupled experiment using an ocean general circulation model (OGCM) with realistic thermohaline circulation.

This experiment must be regarded only as a test of the sensitivity of the ocean circulation to a specified forcing, and not as a prediction.

The model

We use the Hamburg large-scale-geostrophic OGCM⁷. The model is based on the conservation laws for heat, salt and momentum (the latter in a linearized form) and uses the hydrostatic assumption. The flow field is divided into a vertically averaged barotropic component and the residual baroclinic components. The model has a dynamically active sea surface elevation. Changes in the surface elevation are due to divergence of flow, thermal expansion and freshwater fluxes. The formulation of the model is fully implicit and therefore a time step of

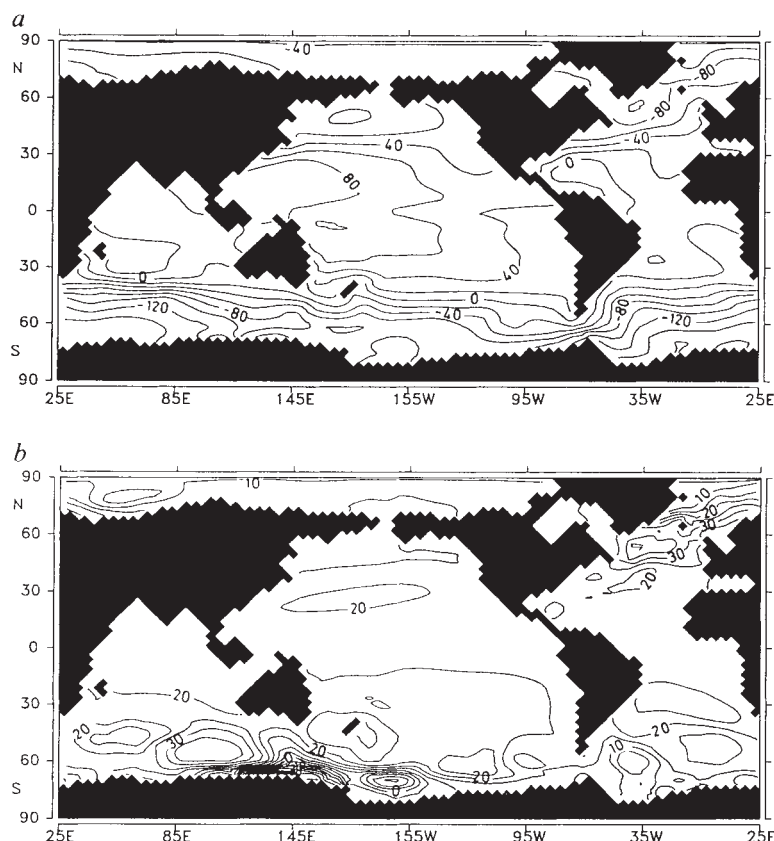


FIG. 1 a, Annual mean distribution of sea surface elevation (cm) of the control experiment. b, Pattern of changes in sea surface elevation (cm) for year 50 relative to the control experiment. Note that changes in the Ross Sea area are negative.

30 days can be used, as compared with a few hours typical for explicit integration schemes. A one-layer thermodynamic sea-ice model with advection is included.

The horizontal resolution is $3.5 \times 3.5^\circ$ on an E-type grid⁸. The model has 11 vertical layers (centred at depths of 25, 75, 150, 250, 450, 700, 1,000, 2,000, 3,000, 4,000 and 5,000 m).

The model was started from a homogeneous state and integrated for 10,000 years to a steady state under climatological forcing (monthly mean wind stress⁹ and air temperature¹⁰ and annual mean climatological surface salinity¹¹) coupled to the top layer with newtonian time constants of ~ 2 months. During the last 500 years of the initial period the freshwater fluxes required to restore the salinity to observed values were stored and used as forcing in a control run, for which the surface salinity was no longer prescribed but calculated from the freshwater fluxes. Initially the model showed a slight drift with this type of forcing, but after a further 4,000 years of integration the model again reached a steady state that was very close to the state attained before switching to freshwater-flux forcing.

The model exhibits a realistic mean thermohaline circulation¹² with a flow of 17 Sv (1 Sverdrup is $10^6 \text{ m}^3 \text{ s}^{-1}$) of North Atlantic deep water (NADW) from the Atlantic at 30°S to the Southern Ocean. At 24°N the strength of the meridional overturning is 20 Sv compared to 17 Sv estimated from tracer distributions¹³. The strength of the Antarctic circumpolar current (ACC) is 122 Sv, compared to 123 ± 10 Sv derived from pressure-gauge measurements¹⁴. The annual mean sea surface elevation of the control run is displayed in Fig. 1a, showing the well-known subtropical gyres and the ACC. The equilibrium circulation fields at the end of this simulation were used as initial conditions in the following experiment.

The experiment

The ocean model was driven by monthly mean anomalies of surface air temperature due to the doubling of atmospheric CO_2 derived from four equilibrium-response experiments conducted with the AGCMs of the Goddard Institute for Space Studies (GISS), Geophysical Fluid Dynamics Laboratory (GFDL), Oregon State University (OSU) and United Kingdom Meteorological Office (UKMO)¹⁵⁻¹⁸. All AGCMs were coupled with mixed-layer oceans. Rather than using the results from a single model, surface air temperature changes for the four models were interpolated to the OGCM grid and averaged. The resulting model average anomaly fields, defined as an annual cycle over 12 months, use all available published equilibrium-response results with comparable levels of atmosphere-ocean interaction.

The patterns of surface temperature changes were very similar for the four models, with poleward amplification of the temperature change in the winter hemisphere owing to ice-albedo feedback effects. The principal differences between models were in the amplitudes of the surface temperature changes. Thus our averaging procedure should not significantly smooth the

response patterns but should be less biased by individual model sensitivities. The average of the four monthly mean anomaly fields was positive everywhere with a global mean warming of 4°C over the oceans. The annual mean spatial pattern of the applied temperature anomaly is shown in Fig. 2.

We assumed that the model-average patterns are time-invariant, with only the amplitude varying during the transition from the control run to doubled atmospheric CO_2 . To simulate the expected transient temperature increase in response to time-dependent forcing, the amplitude evolution was described by the function $f(t) = 1 - e^{-(t-t_0)/a}$ with a time constant a of 40 yr. The initial time t_0 was set to 1 January in year 0. By the year 50 the projected warming is 2.9°C . The forcing that might occur because of changes in the wind stress and freshwater fluxes relative to the control run was not considered.

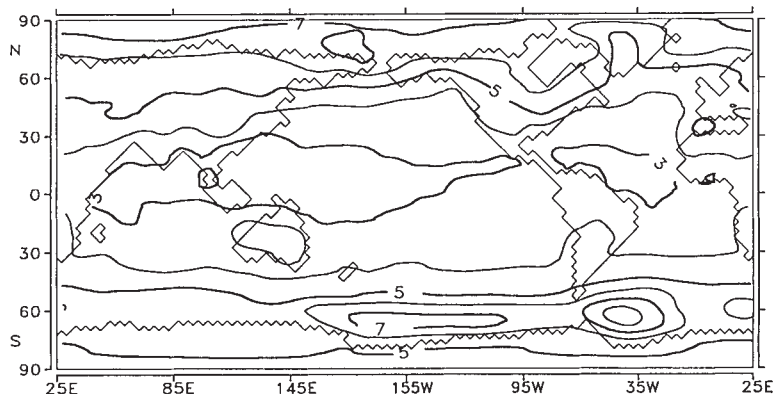
The neglect of atmospheric feedback, anomalous wind stress and freshwater-flux forcing, and the assumption of time-invariant patterns of the surface temperature change in the forcing fields clearly represent major idealizations in our experimental design. Recent evidence from the few GCM experiments that have been performed with time-dependent forcing suggests that important differences may exist between the equilibrium and transient response patterns^{1,3,19}. We feel, however, that our experiment, despite its simplifications, can give valuable insight into typical properties of sea-level response which may not have been generally appreciated and which would also apply to a more realistic coupled ocean-atmosphere.

Results

The time evolution of the deep-water production is shown in Fig. 3a. It is calculated as the rate of potential energy loss due to convection. The global deep-water production decreases initially and attains a minimum in year 40 (approximately two-thirds of its original value). The fractional reduction is stronger in the North Atlantic than in the Southern Ocean. After year 40, the global deep-water formation increases again. This can be attributed exclusively to increased formation of Antarctic bottom water (AABW). Generally there is a trend in the deep-water formation to a higher fraction of AABW compared to NADW.

Figure 4 displays the changes in sea surface temperature (SST) relative to the control for year 50. Although the average surface air temperature anomalies are positive everywhere at all times, with the largest values in the winter hemisphere at ice margins (Fig. 2), negative SST anomalies appear in the vicinity of the Irminger Sea and in the Weddell Sea. This pattern of local SST decrease (or much weaker increase than the rest of the ocean) is persistent throughout the experiment, with the largest decreases occurring in the Irminger Sea. This is the region in which the model produces most of its NADW in the control run. The result can be attributed to the suppressed production of deep water, and to a resultant reduction in the large convective

FIG. 2 Pattern of the annual mean surface air temperature anomaly (the average of four models) used as forcing in the simulation. The global mean of this temperature anomaly is 4°C (calculated over ocean points only).



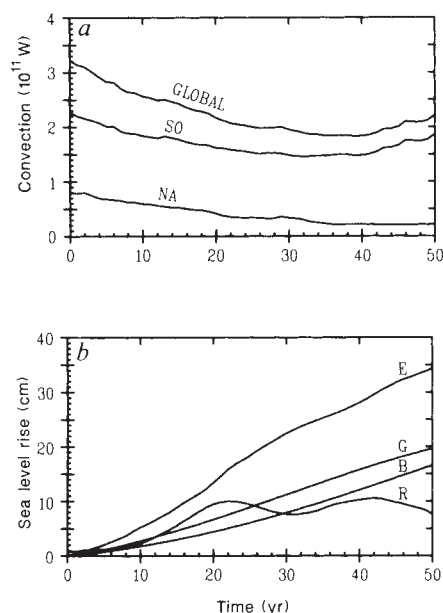


FIG. 3 *a*, Time evolution of global, North Atlantic (NA) and Southern Ocean (SO) deep-water formation, measured as rate of loss of potential energy due to convection. *b*, Sea level rise as a function of time: global mean (G), northwest Europe (E), Ross Sea (R) and Bay of Bengal (B).

heat flux from lower layers to the surface, which in the control run was responsible for the relatively warm temperatures in this region. The cooling process is accompanied by a freshening of surface waters in the North Atlantic and higher surface salinities at lower latitudes (Fig. 5).

A section through the western Atlantic (Fig. 6) shows the penetration of the warming signal into the deep ocean for year 50. Except for polar regions, where the suppression of the upward deep convective heat flux is an efficient mechanism for warming the deep ocean, warming of 0.2°C or more is confined to the upper 1,300 m. In the deep Atlantic, a cooling is seen. This is the result of an increase in the contribution of cold fresh AABW relative to that of warm salty NADW.

The time evolution of the global mean sea level rise caused by thermal expansion is shown in Fig. 3*b*. In year 50 the global mean sea level rise is 19 cm. This is almost completely due to effects above the thermocline. The steady-state sea level rise after 2,000 years of integration is 51 cm. Whereas the prescribed atmospheric warming in year 50 has reached 72% of the equilibrium value, the response in global mean sea level is only 38.4%. Eighty years were required for the sea level to reach 50% of its equilibrium level, and 210 years to reach 75%. This clearly

demonstrates the long timescales involved in the downward penetration of the warming signal.

The curves for selected locations differ considerably (Fig. 3*b*). The sea level increase of 35 cm in northwest Europe is almost twice as large as the global mean sea level change, whereas the increase in the Ross Sea is much smaller than the global mean. In general, the local sea level changes owing to changes in the ocean circulation pattern are of the same magnitude as the changes from local thermal expansion, and both vary regionally.

The geographical distribution of the sea level change for the year 50 is shown in Fig. 1*b*. The largest increases in sea level occur in the North Atlantic, as a result of the reduced NADW formation and reduced surface density, and on the northern edge of the ACC, because of its southward displacement. The increased gradient of sea level across the ACC is due to the larger fraction of bottom water produced at this location, as expressed by the increased vertical homogeneity of the properties of this water mass.

Discussion

Coupled ocean-atmosphere models have not been used to investigate the sea-level response to greenhouse-gas forcing¹⁻³. We are therefore restricted to comparing our sea level changes with results from simpler models.

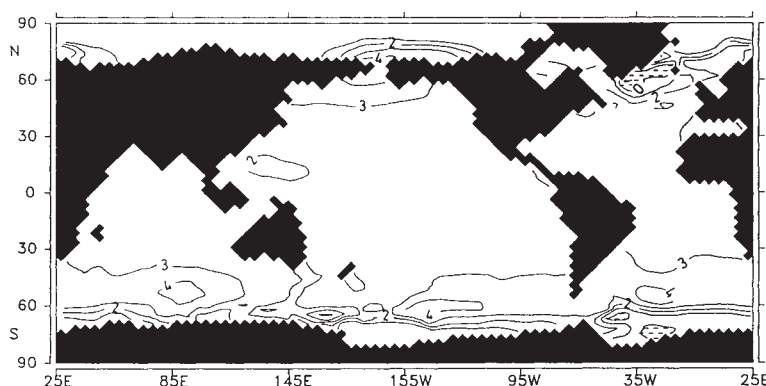
Wigley and Raper²⁰ used an upwelling diffusive energy-balance model to estimate the global sea level rise from thermal expansion alone. For a comparable climate sensitivity ($\Delta T_{2\times} = 4.5^\circ\text{C}$) to that used here and best-guess model parameters, they obtained an estimated global sea level rise of 23 cm between 1985 and 2050. This is in good agreement with the global mean value obtained here (19 cm after 50 years).

With a simple model based on linear concepts of ventilation of the thermocline and ocean-current balance, Godfrey *et al.*⁶ arrived at a global sea level rise due to thermal expansion of between 13 and 30 cm after 60 years for an assumed global warming of 3°C in the first 40 years. This model also provides information on the spatial patterns of projected changes in sea level. They obtain an almost uniform increase, with slightly weaker values around Antarctica. The disagreement with our results can be attributed to their neglect of changes in the deep-water formation and the associated changes in the convective heat flux, which we found to be the most important effect in our experiment.

The circulation changes obtained here can be compared with those obtained in recent experiments with coupled ocean-atmosphere GCMs.

A characteristic feature of our experiment, the cooling or reduced warming of SST in zones where deep water is produced, has also been found in a coupled AGCM-OGCM simulation with sector geography¹. The model's equilibrium response to the doubling of atmospheric CO_2 yielded a cooling around Antarctica, with maxima located at the positions of strongest deep-water formation in the control experiment. Owing to an

FIG. 4 Geographical distribution of changes in SST in year 50 relative to the control experiment. Shading indicates negative values.



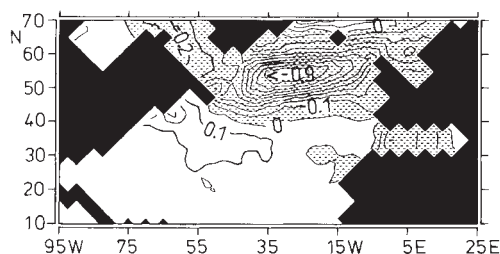


FIG. 5 Geographical distribution of changes in the surface salinity in the North Atlantic in year 20 relative to the control experiment. Shading indicates negative values.

almost complete lack of NADW formation in this simulation, however, there was no equivalent cooling in the Northern Hemisphere.

The results of a recent coupled AGCM-OGCM³ simulation with a transient CO₂ increase of 1% per year (compound) are similar to ours. After 60 years of integration, the production of AABW is increased relative to NADW and in the zonal average a cooling is found in the deep ocean centred at 30° N, as also seen in Fig. 6. There is a relatively weak (compared to the zonal average) warming in the North Atlantic and a cooling around Antarctica. This pattern of SST changes is also generally similar to ours, except for a discrepancy between our strong North Atlantic cooling and the weaker warming seen in the experiment of Stouffer *et al.*³. This discrepancy may be related to differences in the production of NADW, which is considerably weaker in their control run than in ours. Surface freshening in the North Atlantic (Fig. 5) is seen in both simulations. Stouffer *et al.* attribute this to a regional increase in runoff and to the difference between precipitation and evaporation, an explanation that cannot apply here. In our simulation, the weakening of the thermohaline circulation (which reduces northward advection of warm saline surface water from low latitudes and increases exposure time to the climatological net excess of precipitation in high latitudes), together with freshwater input from the melting of sea ice, are responsible for this effect.

The reduced production of NADW and the related cooling offers a possible explanation for the observed trend of decreasing marine air temperatures in the North Atlantic during the last

20 years, in contrast to the increase in Northern Hemisphere and Southern Hemisphere annually averaged surface air temperature²¹. This is consistent with the observation of a strong negative salinity anomaly in the North Atlantic during the 1970s. This anomaly was accompanied by suppression of convection and surface cooling²². During this period the surface salinity further south (at ~40° N) was increased relative to that in the late 1950s²³. A similar feature is found in the model response (Fig. 5).

The simulated cooling in the deep Atlantic is consistent with the trend inferred from a comparison of temperature data taken in 1957 and 1981 for two trans-Atlantic sections at 24° and 36° N (ref. 24). Although there are some striking similarities between the observed changes from the late 1950s to the 1970s and early 1980s and the pattern that the model produces in response to the imposed warming, the observed anomalies could well be generated by other effects—for example, anomalous atmospheric circulation patterns²⁵. In the 1980s the surface salinity in the North Atlantic was returning to its former values²² and the relative cooling trend abated.

Conclusion

Significant changes are induced in the ocean circulation in response to an imposed forcing in surface air temperature corresponding to GCM equilibrium-response results for doubled atmospheric CO₂. In regions where deep-water formation was formerly active, the projected increase in SST is small or even decreases relative to the zonal average. The formation of NADW is more strongly suppressed than the formation of AABW. The subsequent changes in deep-water temperatures and salinities result in an effective cooling of the deep Atlantic. This signal may be useful in the detection of CO₂-induced climate change, although signal-to-noise problems are even more serious in the deep ocean than for surface data owing to the sparseness of observations. Recently, the measurement of acoustic travel times has been proposed as a technique for monitoring deep-ocean temperature changes associated with a CO₂ warming²⁶. Our results stress the need for reliable studies of ocean circulation response to calculate the magnitude (and even sign) of the expected temperature changes.

The projected atmospheric warming of ~3 °C by year 50 leads to a global sea level rise of 19 cm from thermal expansion alone. But this is only one component of the simulated sea-level response. Regionally specific changes are of the same magnitude,

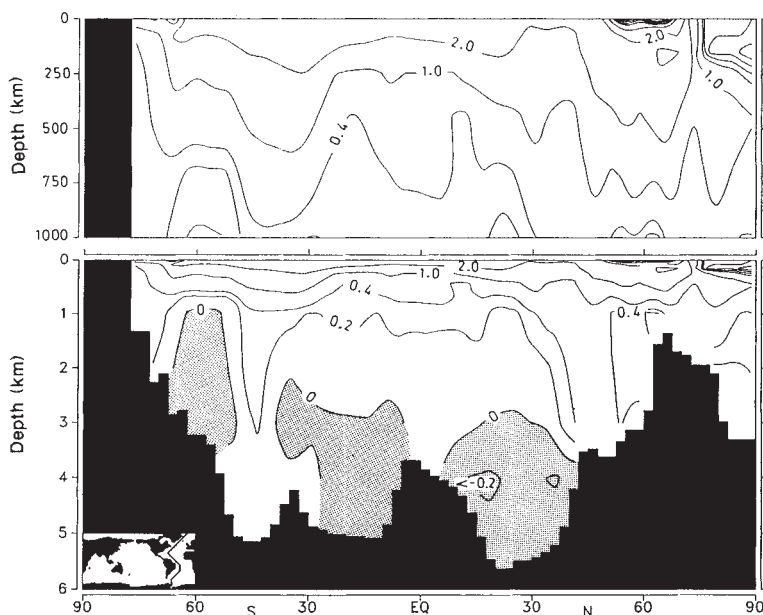


FIG. 6 Change of potential (with respect to surface pressure) temperature with depth along a meridional section through the Atlantic in year 50 relative to the control experiment. The location of the section is shown in the insert. Shading indicates negative values.

with the strongest increases predicted in the North Atlantic, and the weakest increases (or even a decrease) predicted for the Ross Sea. Simpler models without a dynamic ocean cannot treat such effects in a realistic way. This shows the need for using an OGCM with realistic ocean circulation (particularly for the deep ocean) to simulate the regional sea level changes in response to climate forcing.

The decrease in global deep-water formation rates must be

expected to have a strong impact on the atmospheric CO₂ content. At present, a significant fraction of the emitted CO₂ is transported to the deep ocean by the newly formed deep water, thus limiting the increase in atmospheric p_{CO_2} (ref. 7). If the efficiency of this marine transport and storage mechanism is reduced, the increase in atmospheric p_{CO_2} will be amplified. This positive feedback can have important consequences for the evolution of future atmospheric CO₂ concentrations. □

Received 19 February; accepted 23 April 1990.

1. Bryan, K., Manabe, S. & Spelman, M. J. *J. phys. Oceanogr.* **18**, 851–867 (1988).
2. Washington, W. & Meehl, G. *Clim. Dynamics* **2**, 1–38 (1989).
3. Stouffer, R. J., Manabe, S. & Bryan, K. *Nature* **342**, 660–662 (1989).
4. Gornitz, V., Lebedeff, S. & Hansen, J. *Science* **215**, 1611–1614 (1982).
5. Wigley, T. M. L. & Raper, S. C. B. *Nature* **330**, 127–131 (1987).
6. Godfrey, J. S., Church, J. A., Jackett, D. R. & McDougall, T. J. *Nature* (submitted).
7. Maier-Reimer, E. & Hasselmann, K. *Clim. Dynamics* **2**, 53–90 (1987).
8. Arakawa, A., Lamb, V. R. *Meth. comput. Phys.* **16**, 173–263 (1977).
9. Hellerman, S. & Rosenstein, M. *J. phys. Oceanogr.* **13**, 1093–1104 (1983).
10. Woodruff, S. D., Slutz, R. J., Jenne, R. L. & Steurer, P. M. *Bull. Am. met. Soc.* **68**, 1239–1250 (1987).
11. Levitus, S. *Climatological Atlas of the World Ocean*, Prof. Pap. 13 (NOAA, Rockville, 1982).
12. Maier-Reimer, E. & Mikolajewicz, U. In *Oceanography 1988* (eds Ayala-Castan˜ares, A., Wooster, W. & Yáñez-Arancibia) 87–100 (UNAM-Press, Mexico DF, 1990).
13. Roemmich, D. & Wunsch, C. *Deep Sea Res.* **32**, 619–664 (1985).
14. Whitworth, T. & Peterson, R. G. *J. phys. Oceanogr.* **15**, 810–816 (1985).

15. Hansen, J. et al. in *Climate Processes and Climate Sensitivity* (eds Hansen, J. & Takahashi, T.) 130–163 (Am. geophys. Un., Washington, DC, 1984).
16. Wetherald, R. Y. & Manabe, S. *Clim. Change* **8**, 5–23 (1986).
17. Schlesinger, M. E. & Zhao, Z.-C., *J. Clim.* **2**, 459–495 (1989).
18. Wilson, C. A. & Mitchell, J. F. B. *J. geophys. Res.* **92**, 13315–13343 (1987).
19. Hansen, J. et al. *J. geophys. Res.* **93**, 9341–9364 (1988).
20. Wigley, T. M. L. & Raper, S. C. B. in *Climate and Sea Level Change: Observations, Projections and Implications* (eds Warrick, R. A. & Wigley, T. M. L.) (Cambridge University Press, 1990).
21. Jones, P. D., Wigley, T. M. L. & Farmer, G. in *Proc. DOE Workshop on Greenhouse-Gas-Induced Climatic Change* (ed. Schlesinger, M. E.) (Elsevier, Amsterdam, 1990).
22. Dickson, R. R., Meincke, J., Malmberg, S.-A. & Lee, A. J. *Prog. Oceanogr.* **20**, 103–151 (1988).
23. Levitus, S. *J. geophys. Res.* **94**, 9679–9685 (1989).
24. Roemmich, D. & Wunsch, C. *Nature* **307**, 446–450 (1984).
25. Dickson, R. R., Lamb, H. H., Malmberg, S.-A. & Colebrook, J. M. *Nature* **256**, 479–482 (1975).
26. Munk, W. H. & Forbes, A. M. G. *J. phys. Oceanogr.* **19**, 1765–1778 (1989).

ACKNOWLEDGEMENTS. We thank K. Hasselmann, T. Wigley and S. Levitus for discussions and M. Grunert for preparing the figures.

Three-dimensional structure of the free radical protein of ribonucleotide reductase

Pär Nordlund, Britt-Marie Sjöberg* & Hans Eklund

Department of Molecular Biology, Swedish University of Agricultural Sciences, Biomedical Center, Box 590, S-75124 Uppsala, Sweden

* Department of Molecular Biology, University of Stockholm, S-10691 Stockholm, Sweden

The enzyme ribonucleotide reductase furnishes precursors for the DNA synthesis of all living cells. One of its constituents, the free radical protein, has an unusual α -helical structure. There are two iron centres that are about 25 Å apart in the dimeric molecule. Tyrosine 122, which harbours the stable free radical necessary for the activity of ribonucleotide reductase, is buried inside the protein and is located 5 Å from the closest iron atom.

RIBONUCLEOTIDE reductase is a key enzyme in all proliferating cells. It catalyses the *de novo* production of deoxyribonucleotide precursors for DNA synthesis. In *Escherichia coli* the reduction of ribonucleotides takes place at the diphosphate level and in this and several other respects this enzyme is similar to mammalian and virus-induced ribonucleotide reductases¹. Because of its importance in DNA synthesis, ribonucleotide reductase is a potential target for drug design, particularly for the development of antiviral agents.

E. coli ribonucleotide reductase contains α - and β -subunits: the holoenzyme is $\alpha_2\beta_2$. The two dimeric forms α_2 and β_2 are known as protein B1 and protein B2 respectively. The large α -subunits contain the substrate-binding sites, but both B1 and B2 contribute to the active site of the enzyme. B2 contains an essential tyrosyl radical which is stabilized by an adjacent dinuclear iron centre and is thought to initiate the radical-based reaction. In a subsequent step, redox-active cysteines in B1 provide the electrons for the reduction of ribose.

The first example of a stable free radical in proteins was found in B2 from *E. coli*², but they are also present in ribonucleotide

reductases from other species^{3–5}. Electron spin resonance (ESR) spectroscopy has been used to characterize the radical as a protein-derived tyrosyl radical in the vicinity of the iron centre³. Site-directed mutagenesis has shown that the radical is located at Tyr 122 (ref. 6). Tyrosyl radicals were subsequently found in other proteins such as photosystem II (ref. 7), prostaglandin H synthase⁸ and galactose oxidase⁹. Here we present the crystal structure of the *E. coli* ribonucleotide reductase B2 protein determined at 2.2 Å resolution.

The B2 structure

Details of the structure determination are given in Table 1. Helices make up about 70% of the B2 subunit, which has only one pleated-sheet region, from residues 173–184, which forms an antiparallel hairpin. The α -helical structure of the B2 subunit is in a novel arrangement having an eight-helix bundle as the main motif. The helices in the bundle (A to H; Fig. 1a) are quite long, four of them comprising 30 residues or more. The proteins most similar to B2 in this respect are bacteriorhodopsin¹⁰ and the photosynthetic reaction centre¹¹. B2, however, is not a membrane protein. A large part of the subunit consists of a barrel of helices with the very long hydrophobic helix α E in the middle, surrounded by five helices (Fig. 1b). Comparably long hydrophobic helices exist in cytochrome P₄₅₀ (ref. 12) and citrate synthase¹³. The α -barrel radius of B2 is smaller than in an α/β barrel, whose centre is formed by eight β -strands.

The subunit can be described as having three layers of helices (Fig. 2). The B2 dimer is formed mainly by homologous interactions between the first layer of the helices of each subunit around the molecular twofold axis. The helices at the subunit-subunit interface cross at an angle of $\sim 140^\circ$ relative to one another: the tilted interaction makes the molecule heart-shaped (Fig. 3). One exceptional feature of the molecule is the extension at the bottom

of the heart. This is formed by the four-stranded antiparallel sheet, with two strands from each subunit.

The iron centre

Ever since the iron centre in B2 was first reported¹⁴, it has been assumed that there is only one iron centre per dimer. Recently it was suggested¹⁵, on the basis of improved metal analysis and other investigations, that the B2 dimer contains two iron centres. The three-dimensional structure of the B2 dimer unambiguously shows that it does contain two equivalent iron centres, and their location was confirmed from electron density maps of crystals of the iron-deficient form, apoB2 (ref. 16). The difference map at 4.5 Å resolution between apoB2 and native B2 showed two elongated negative peaks (9σ) at the positions of the iron centres. The distance between the two iron centres is 25 Å, and 3.3 Å between the two iron atoms, Fe1 and Fe2, within each iron centre. The iron atoms are ligated to four helices, B, C, E and F, two of which form the central part of the dimer interface. Several properties of the protein can now be explained that before were difficult to reconcile with only a single iron centre at the interface between the two β -subunits^{1,17,18}.

The quality of the refined structure ($R = 0.20$ at 2.2 Å resolution) allows a detailed description of the iron-centre geometry (Fig. 4). One glutamic acid residue at position 115 is ligated to both iron atoms, forming a bridge between them. A second bridge is formed by a non-protein ligand, which became visible

FIG. 1 *a*, Schematic drawing of the secondary structure elements of the B2 subunits. Helices in the main helical bundle are labelled with letters, and remaining helices with numbers. All helices are drawn as cylinders. Amino acids in the secondary structure elements are: α A: 34–46; α 1: 55–65; α B: 66–96; α C: 101–130; α 2a: 133–143; α 2b: 143–151; α D: 152–170; β 1: 173–178; β 2: 179–184; α E: 185–223; α F: 224–254; α 3: 258–268; α G: 268–291; α H: 300–320; α 4: 332–339. The two iron atoms are depicted as small spheres. Helix α 2, which is broken in the middle, is partly hidden. *b*, Core helices of the B2 subunit, emphasizing the α -barrel structure, with a central helix, α E, surrounded by five other helices. The B2 α -barrel is not as regular as an α/β barrel and does not show an approximate fivefold symmetry. The helices are coloured as follows: α B, yellow; α D, brown; α E, light green; α F, red; α G, violet; α H, blue. A sixth helix, α C, could also be included in the α -barrel, but is not, because the iron centre prevents extensive contacts with helix α E.

during refinement. This ligand has been shown by resonance Raman spectroscopy to be O^{2-} (μ -oxo bridge) (ref. 19). Glu 238 is bound to Fe2 and points towards Fe1, but it is not close enough for direct coordination to Fe1. Fe1 is also ligated to His 118 and Asp 84, and Fe2 to His 241 and Glu 204. Two other non-protein ligands, one for each iron atom, appeared during refinement and we have interpreted these as being water molecules.

Fe1 has a coordination with both trigonal bipyramidal and octahedral features, due to the fact that both carboxylate oxygen atoms of Asp 84 bind to Fe1. The distorted plane is completed by His 118 and the O^{2-} . Perpendicular to this plane, the iron atom is ligated by Glu 115 and a water molecule. Fe2 has a regular octahedral coordination, with angles close to 90°. The μ -oxo bridge is *cis* with respect to the histidines and is not in the *trans* configuration proposed earlier^{20–21}.

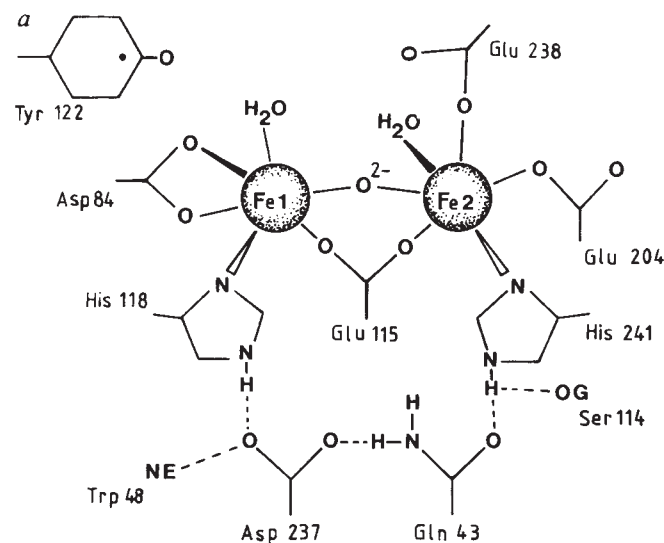


FIG. 2 *a*, Schematic drawing of the folding within the three layers of helices that form one subunit of B2. Each helix is drawn as a cylinder, each iron atom as a small sphere, and the ligands as lines connecting the irons to the helices. Helices F, E and G in the second layer should be superimposed on top of helices C, B and D in the first layer and helix H in the third layer superimposed on helix G. The helix α B contributes the ligand Asp 84, α C, Glu 115, His 118 and the free radical tyrosine, α E, Glu 204, and α F, Glu 238 and His 241. *b*, Schematic drawing of the fold of the subunit looking along the helices A–H. The small circles in the centre of the subunit are the two iron atoms.

FIG. 4 *a*, Schematic drawing of the coordination of iron atoms at each iron centre. Residues hydrogen-bonded to iron ligands and residues hydrogen-bonding to these residues are also included. Tyr 122 is close to the iron centre but is not directly coordinated to it. *b*, The iron centre of subunit 1 with the electron density map in light blue. The water molecule on Fe2 to the left is pointing towards the viewer, the water molecule on Fe1, to the right, is pointing upwards. *c*, Model building of molecular oxygen bound to Fe1. The oxygen molecule with its atoms in red spheres is coordinated to Fe1 in the centre of the picture (pink sphere). The front of the picture consists of Fe1 and its ligand Asp 84. The oxygen molecule projects backwards into a pocket created by Tyr 122, Phe 208, Phe 212, Ile 234 and Glu 238 (a ligand to Fe2) in a blue-dotted surface of van der Waals' radii.

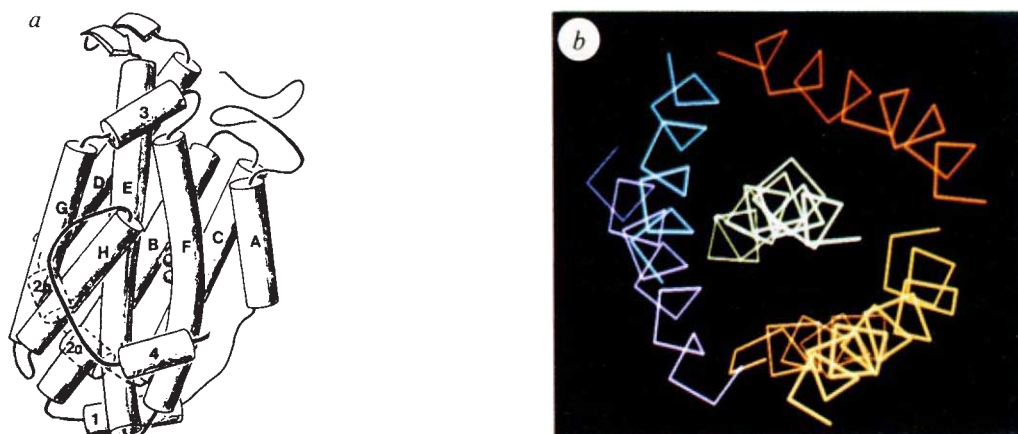
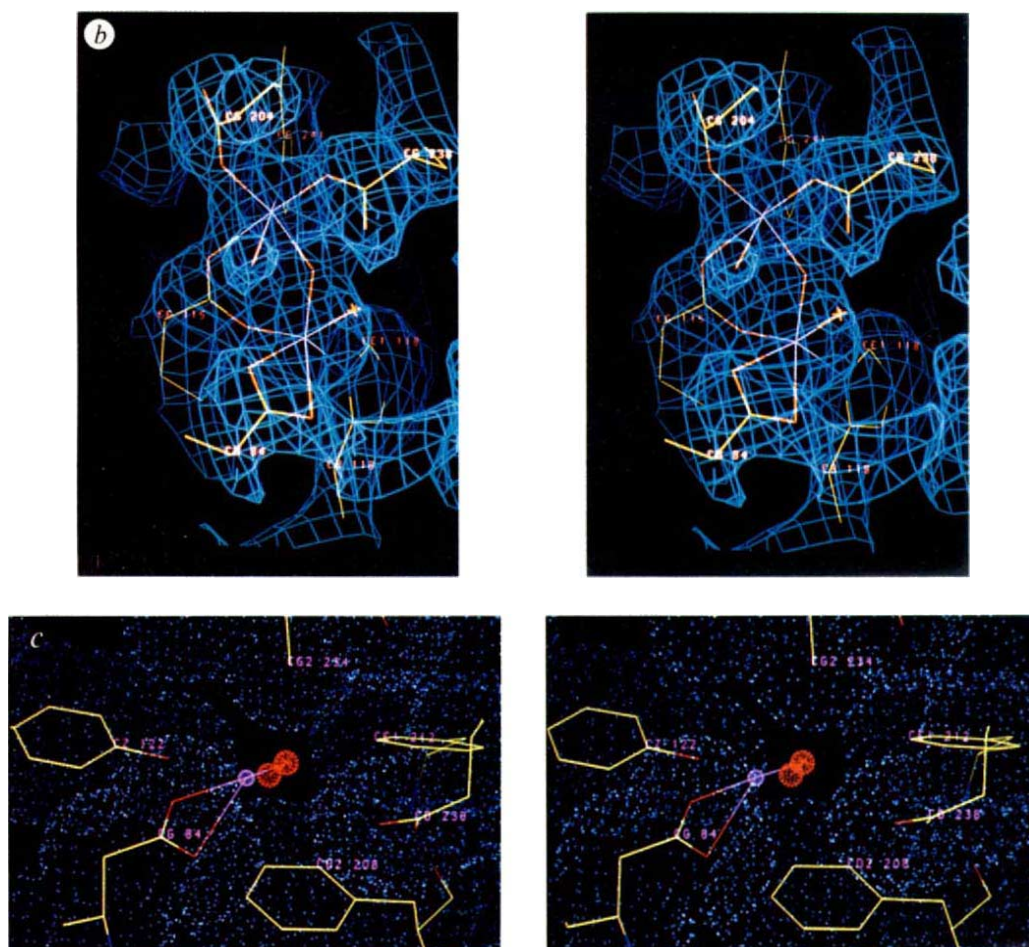
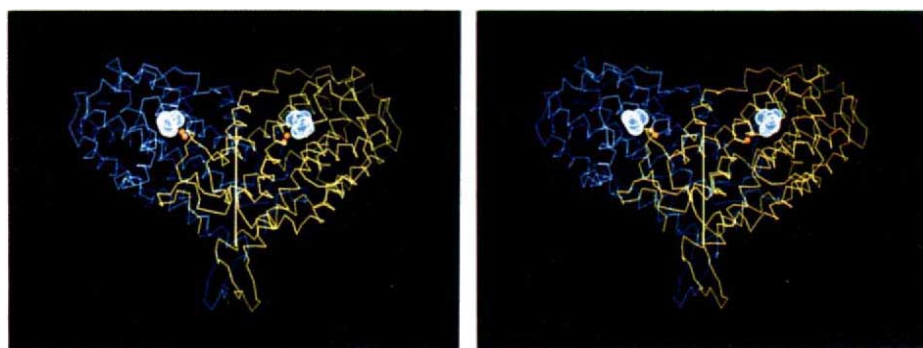


FIG. 3 The B2 dimer with the $C\alpha$ chain in one subunit in blue and in the other subunit in yellow. The iron atoms of the two iron centres are shown as orange spheres. The van der Waals' surface of Tyr 122 of each subunit is shown in light blue. The molecular twofold axis is drawn as a vertical yellow bar.



Each ferric ion is liganded by a histidine N δ atom. The histidines are both located on the same side of the binuclear iron centre with their C ϵ atoms only 4 Å apart and are involved in an extensive hydrogen-bonded network with their N ϵ atoms (Fig. 4). Asp 237 occupies a central position in this network and is hydrogen-bonded to His 118 and Trp 48 through one oxygen atom.

B2 amino-acid sequences from several species have been determined by gene sequencing and found to be phylogenetically related (see refs 47–57). Sequence homology between the different B2 proteins is low, and only 16 residues are conserved within all species. Most of the conserved residues are at the iron centre, or close to it. These include all iron ligands except Asp 84, which is a glutamic acid in B2 of the Epstein–Barr virus, and residues in the hydrogen-bonded network. Tyr 122 is conserved in all species, as are some hydrophobic residues close to the OH of Tyr 122 (Fig. 4c).

Duplication of an iron-binding motif

The iron-binding structure in B2 is built up from two segments, 66–130 and 185–254, each of which contains two helices. The arrangement has quasi-twofold symmetry, with an axis between the iron ions perpendicular to the helices. The first helix in each segment contributes one carboxyl ligand (Fig. 5). The second helix in each segment contains one carboxyl ligand and one histidine ligand. The two segments superimpose closely, with a root-mean-square difference of 1.4 Å for C α of 60 residues. Using the same transformation matrix, helices D and G and the intervening helices 2 and 3 also roughly superimpose, indicating that the duplicated motif is in fact a larger unit of ~110 residues.

We find sequence homology between the two segments in each species which is indicative of a gene duplication (Fig. 5). The internal homology is most pronounced in the vicinity of the carboxyl ligands 84 and 204 in helices B and E. Circumstantial evidence supporting a gene duplication is also found in the

mouse B2 gene, where one of the introns²² is located between the two duplicated motifs. Gene duplication as a mechanism for forming multiple metal-binding sites has been proposed for other metal-binding proteins^{23–26}.

Comparison with haemerythrin

In the absence of a crystallographically determined B2 structure, the structure of the iron-containing protein haemerythrin has often been used as a model for B2. The absorption, Mössbauer, ¹H NMR and resonance Raman spectra of these proteins have some similarities²¹. The four central helices in B2 that bind the ferric ions form a haemerythrin-like structure embedded in the rest of the subunit. However, the iron-binding motifs in haemerythrin and B2 differ in detail: although the binuclear iron centres superimpose, the axis through the two iron atoms is roughly perpendicular to the helices in haemerythrin and is essentially parallel to them in B2; also, the order of the helices is different. We conclude that there is no obvious evolutionary connection between the two proteins.

The iron ligands are different in B2 and in haemerythrin. The iron centre in haemerythrin is ligated to five histidines, two bridging carboxyl acids, and one bridging O²⁻ ion^{27,28}. Furthermore, in haemerythrin, histidine N ϵ atoms are bound to the ferric ions, in contrast to B2, where the N δ atoms are the histidine ligands. Several iron proteins have mixed nitrogen-oxygen ligation, but apart from B2, only the transferrins have predominantly oxygen ligands²⁴.

Free-radical-harboursing Tyr 122

The tyrosyl radical in active B2 is stable for more than a week at 4 °C. Inactivation involves a gradual reduction to a normal tyrosine while the iron atoms remain oxidized: this form is called metB2. Spectroscopy of single crystals of B2 have shown that they consist of metB2 (ref. 16). The coordination of the iron atoms in active B2 and metB2 is probably the same^{20,30,31}.

TABLE 1 Data collection and refinement statistics

Compound	Resolution (Å)	Unique reflections (% of total)	Merging <i>R</i> (%) [*]	Isomorphous difference (%)	Number of sites [†]	Refinement statistics	
						R.m.s. <i>f</i> _H /r.m.s. <i>ε</i> (15–3.5 Å)	Centric <i>R</i> (%) [‡]
Native	2.2	74	5.9				
PtCl ₆ ²⁻	3.0	89	7.0	13.6	8	1.35	57
Pt(NH ₃) ₂ Cl ₂ (<i>cis</i>)	3.0	70	4.6	13.5	11	1.29	58
Pb(OOC.CH ₃) ₂	3.5	84	5.9	17.6	16	1.05	63
PbCl ₂	3.1	86	5.1	13.6	16	1.39	58
Pt(NO ₂) ₄ ²⁻	3.1	87	5.9	18.2	9	1.10	62

^{*} Merging $R = \sum |I - \langle I \rangle| / \sum I$.

[†] This number also includes the native mercury sites with changed occupancies.

[‡] f_H , Heavy atom vector; ϵ , lack of closure; R centric = $\sum |f_H - \epsilon| / \sum |f_H|$.

Crystals: Crystals of protein B2 from *E. coli* were obtained using 20% PEG 4000, 0.2 M NaCl, 0.3% dioxane and 0.05 M of the mercury-containing compound ethyl mercuric thiosalicylate in 50 mM MES buffer, pH 6.0, as described¹⁶. These crystals are orthorhombic and contain one dimer (2 × 375 residues, with a relative molecular mass of 43,300) per asymmetric unit. Protein B2 was purified from an overproducing strain¹⁷. Crystals belong to space group *P*2₁2₁2₁, with cell-dimensions 74.3, 85.5 and 115.7 Å. **Data collection and scaling:** X-ray diffraction data sets were collected on a Nicolet multiwire area detector mounted on a Rigaku rotating anode. Reflections were measured in 0.1° oscillation frames and evaluated using the Buddha program²⁹. A full data set was usually collected from one crystal. The CCP4 suite of programs (Daresbury Lab., UK) was used for scaling of measurements. Agreement between symmetry-related reflections (*R* merge) ranged from 4 to 7% for different data sets. **Heavy atom derivatives and phasing:** The PtCl₆²⁻-derivative contained two principal sites that could readily be located in the difference Patterson map. Using these sites and difference-Fourier techniques the heavy atom structures were revealed in detail. For initial heavy-atom refinement, we used the program Heavy⁴⁰. In the final phasing, five heavy atom derivatives were used. The mean figure of merit was 0.84 for reflections between 20 and 4.5 Å, 0.69 for reflections between 4.5 and 3.5 Å, and 0.41 for reflections between 3.5 and 3.0 Å. Solvent flattening was used for initial phase improvement⁴¹. **Non-crystallographic symmetry:** It was not possible to find a significant unambiguous peak in the self-rotation function calculation on native data sets. The non-crystallographic twofold axis could instead be determined from heavy-atom positions. The position of six pairs of heavy atoms superimpose with a root-mean-square deviation of 0.7 Å by a twofold rotation. A program package by Bricogne⁴² was used for cyclic averaging, phase combination and phase extension in the resolution range 4.5–2.7 Å. The final *R* value was 0.23 and the correlation coefficient, 0.90. **Model building:** Skeletonized electron-density maps⁴³ were used to locate helices, to define the two subunits and to investigate connections between helices. The map at 2.7 Å resolution was interpretable in most areas, except in regions involved in crystal packing. FRODO was used for all model-building⁴⁴ and O for structural comparisons⁴⁵. **Refinement:** The coordinates of the B2 model were refined using X-PLOR (ref. 46). The present *R* value for all the reflections in the resolution range 6.5–2.2 Å is 20% (including 150 water molecules). This model contains all residues from 1 to 343. There is residual density for some of the remaining 32 residues in our electron density maps, but no unambiguous chain tracing can be made for them.

Furthermore, the torsion angles of Tyr 122 in our structure are in excellent agreement with those determined from ENDOR measurements³².

The OH of Tyr 122, which harbours the free radical, is 5.3 Å away from the closest iron atom. The radical is located along the axial direction of the two antiferromagnetically coupled ferric ions, where a significant magnetic field should be experienced from the iron centre. This agrees with ESR results showing that the magnetic dipole of the radical is coupled to the magnetic field of the iron centre³³. The tyrosine side chain is in van der Waals' contact with the side chains of residues Leu 77, Gln 80, Thr 81, Asp 84, Ile 234 and main-chain atoms of residues 80, 118, 119 and 123. The closest atom to the OH of Tyr 122 is the oxygen atom of Asp 84, which is 3.3 Å away. A hydrogen bond cannot form between these side chains in the active form of B2 because the radical tyrosine is deprotonated^{20,32}.

B2 can be reactivated *in vitro* by reduction of the ferric ions and subsequent oxidation by molecular oxygen^{3,34,35}, reactions that are also possible in the crystals (P.N., unpublished results). An enzymatic radical-generating system has been described in *E. coli*^{35,36}. The tyrosyl radical can also be formed by incubation of metB2 with hydrogen peroxide in a reaction that is probably similar to the dioxygen-mediated activation of reduced B2 (ref. 37).

There is a binding pocket between Fe1 and Tyr 122 suitable for occupation by a dioxygen species during generation of the tyrosyl radical. Model-building shows that an oxygen or peroxide molecule, when bound to Fe1 at the observed water-ligand position, will reach the tyrosine OH-group (Fig. 4c). This provides a structural basis for the proposed activation mechanism of B2, which involves simultaneous oxidation of the two irons and generation of the oxidized tyrosyl radical^{34,37}.

The remarkable stability of the tyrosyl radical of ribonucleotide reductase has been an enigma. From the structure of B2 it is clear that Tyr 122 is embedded in the protein and is inaccessible to surrounding solvent. It is ~10 Å from the nearest surface (the molecular surface, described later, that is supposed to bind B1). The side chain of Tyr 122 is not in van der Waals' contact with any oxidizable side chain; moreover, the radical is delocalized over the side chain, which makes it less reactive.

The B1 interaction area

Both B1 and B2 are homodimeric proteins and the holoenzyme has two active sites at the interface between B1 and B2. It is therefore likely that B2 interacts with B1 through one of its

surfaces around the twofold axis. On one side of the twofold axis of B2 there is a protrusion (see Fig. 3, bottom) that is formed mainly by the β -sheet. Corresponding regions are not present in sequences other than those of the B2 protein from *E. coli* and bacteriophage T4, and so are unlikely to form the B1 interaction area. The other side of the dimer (Fig. 3, top), which we propose to be the interaction area with B1, forms a flat extensive surface with the conserved residues Trp 48, Asp 58 and Arg 236, plus the highly homologous residues Glu 52, Tyr 307 and Ser 341.

The C terminus of B2 is known to be important in interactions with B1, because a truncated form of B2 without the 30 C-terminal residues cannot bind B1 (ref. 17). Peptides corresponding to the carboxyl end of B2 from herpes simplex virus inhibit the association between the virus-specific B1 and B2 proteins^{38,39}, rendering this side of the molecule a potential target for antiviral drug design. As the last located residue of B2 (343) is in the centre of the proposed interaction area with B1, the C-terminal region of B2 must be located at this side of the molecule, although the structure of the last 32 residues cannot yet be defined. One reason may be that this region of B2 is disordered until the complex with B1 is formed. Investigation of another crystal form of B2 may clarify this point¹⁶; we are also studying crystals of B1-B2 complexes.

How does the buried tyrosyl radical participate in the reduction of ribonucleotides? The generally accepted view is that the tyrosyl radical oxidizes the ribose ring to make it more reactive for the subsequent steps of the reaction. One possibility is that the interaction with B1 changes the local structure around the tyrosine, thus exposing it to the active site; however, ESR spectra of the complex between B1 and B2 are the same as for normal B2, so a conformational change in the vicinity of the Tyr 122 is unlikely³³. A more plausible explanation is that there is electron transfer by a route that might involve Fe1 and an array of conserved residues from the iron centre to Trp 48 at the proposed B1 interaction surface.

Discussion

The structure determination of the stable free radical protein of ribonucleotide reductase has shown that there are two separate iron centres, one for each subunit. The unexpectedly large differences in the iron coordination for B2 and that for haemerythrin indicate that there will not be a general common motif for the formation of binuclear iron centres, and extrapolations from known structures have to be made with great care.

The stable tyrosyl free radical is buried in the protein 10 Å

FIG. 5 Amino-acid sequences from several species of the four helices that have iron ligands. The sequences are arranged in two groups, where each two-helix unit is aligned with the other such that the iron ligands are superimposed. Residues that are identical in both halves are indicated by a black background if they are present in at least six places and at least in two species in each half. The consensus sequences at the bottom of each half have capital letters when present in all species and small letters if present in all sequences but three. Iron ligands are boxed. Amino-acid sequences used are: *E. coli*, *Escherichia coli*⁴⁷; T4, bacteriophage T4⁴⁸; clam, *Spisula solidissima*⁴⁹; Mouse⁵⁰; Yeast, *Saccharomyces cerevisiae*^{51,52}; Vaccinia, vaccinia virus⁵³; HSV2, herpes simplex virus type 2^{54,55}; Varicella, varicella zoster virus⁵⁶; EBV, Epstein-Barr virus⁵⁷.

	<-----αB----->										<-----αC----->									
	70	80	90	100	110	120	130													
<i>E. coli</i>	PEHEK	HIFISN	RYQ	TLL	D	SIQGRS	PNVALLP	IS	...	DE	SDTWET	WAFS	D	TI	H	SR	SYTH	IRNIV	N	
T4	PQYQQ	NIFTNN	RYQ	SLL	D	SIQGRA	PSAVLMS	IS	...	DE	SDTWAT	WIFS	D	TI	H	SR	SYTH	IRNIV	T	
Clam	KKEEK	HFISHV	DAF	AS	D	SIQVEN	LVERFSKEVQ	...	...	VTEARCFYGF	QIAM	D	NI	H	SE	MYS	LIDTYI	K		
Mouse	KPDER	HFISHV	DAF	AS	D	SIQVEN	LVERFSQEVQ	...	...	VTEARCFYGF	QIAM	D	NI	H	SE	MYS	LIDTYI	K		
Yeast	NENER	FFISRV	DAF	AS	D	SIQVEN	LVENFSTEVO	...	...	TEAKSFYGF	QIMI	D	NI	H	SE	TYS	LIDTYI	K		
Vaccinia	TPDER	YFIKHV	DAF	AS	D	SIQVEN	LAERFCTEVQ	...	...	TEARCFYGF	QMAI	D	NI	H	SE	MYS	LIDTYV	K		
HSV2	SEGEL	GHRFL	FAFL	SAA	D	DLVTEN	LGGLSGLPE	...	...	QKDLHYVE	QECI	D	VV	H	SR	VYNI	QLVLF	H		
Varicella	TEDEL	IFRIFITFL	SAA	D	DLVMVN	LGSLTQLFS	...	...	QKDIHYYIE	QECI	D	VV	H	AR	VYSQ	QLVLF	R			
EBV	NERDL	EFKFL	FTFL	AMA	E	KLVNFN	IDELVTSFE	...	...	SHDIDHYTE	QKAM	D	NV	H	GE	TYAN	LNNLF	D		
Consensus	--e--	-f--	-f--	-f--	d	--v--	n	-----	-----	-----	y--	q--	E	--	H	s	-Y	-----		

	<-----αE----->										<-----αF----->									
	190	200	210	220	230	240	250													
<i>E. coli</i>	LRELK	KKLYL	MSV	NAL	E	ALRFYV	SFACSFAPAE	R	ELMEGNA	KIIRL	IARD	D	AL	H	LT	GTQHM	LNIR	S		
T4	KRLLI	KSLYL	QHVY	NAL	E	ALRFYV	SFACTNFHK	N	MEIMEGNA	KIMKF	IARD	D	QL	H	LK	GTQY	IRQIQ	S		
Clam	...DS	SSIAERVVAE	AAV	E	EFPSG	SFASIFW	KK	R	GIMG	UTFSNEL	ISRD	D	GL	H	CD	FACMF	SHV	N		
Mouse	...LKE	ATIGERVVAE	AAV	E	EFPSG	SFASIFW	KK	R	GIMG	UTFSNEL	ISRD	D	GL	H	CD	FACMF	SHV	N		
Yeast	...AD	ALFGERVVAE	ASI	E	EFPSG	SFASIFW	KK	R	GIMG	UTFSNEL	ICRD	D	GL	H	TD	FACMF	FAH	N		
Vaccinia	...DS	AGIGERVVAE	AAV	E	EFPSG	SFASIFW	KK	R	GIMG	UTFSNEL	ISRD	D	GL	H	CD	FACMF	FKH	H		
HSV2	...EC	DSVPEK	FILM	ILI	E	SVFFAA	SFAAIAYRT	N	NLLRV	TQCSNDL	ISRD	D	AV	H	TT	ASCY	YNNYL	G		
Varicella	...DW	PSVAEK	YILM	ILI	E	EFVFS	SFAAIAYRN	N	GLFVV	TQCFNOL	ISRD	D	AI	H	TS	ASCC	YNNYV	P		
EBV	...AA	VTSPEK	ILV	LLI	E	EFVFS	SFYSIALRV	R	GLMG	GICLANNY	ISRD	D	LL	H	TR	AAS	LYNSMT	A		
Consensus	-----	---	E	giff--	SFA-i	-----	---	m-g	---	n-l	I-RD	E	-l	H	---					

from the surface and is protected from reducing molecules, which means that the tyrosine cannot participate in direct hydrogen abstraction from the substrate. Instead, the structural

arrangement in the vicinity of the radical site is indicative of a long-range electron transfer process, which might also involve the iron centre. □

Received 28 December 1989; accepted 19 April 1990.

- Stubbe, J. A. A. *Rev. Biochem.* **58**, 257–285 (1989).
- Ehrenberg, A. & Reichard, P. *J. biol. Chem.* **247**, 3485–3488 (1972).
- Gräslund, A., Sahlin, M. & Sjöberg, B.-M. *Envir. Hlth Perspect.* **64**, 139–149 (1985).
- Harder, J. & Follmann, H. *FEBS Lett.* **222**, 171–174 (1987).
- Ingemarsson, R., Gräslund, A., Darling, A. & Thelander, L. *J. Virol.* **63**, 3769–3776 (1989).
- Larsson, Å. & Sjöberg, B.-M. *EMBO J.* 2037–2040 (1986).
- Debus, R. J., Barry, B. A., Sithole, I., Babcock, G. T. & McIntosh, L. *Biochemistry* **27**, 9071–9074 (1988).
- Karthein, R., Dietz, R., Nastainczyk, W. & Ruf, H. H. *Eur. J. Biochem.* **171**, 313–320 (1988).
- Whittaker, M. M., DeVito, V. L., Asher, S. A. & Whittaker, J. W. *J. biol. Chem.* **264**, 7104–7106 (1989).
- Henderson, R. & Unwin, P. N. T. *Nature* **257**, 28–32 (1975).
- Deisenhofer, J., Epp, O., Miki, K., Huber, R. & Michel, H. *Nature* **318**, 618–624 (1985).
- Poulos, T. L., Finzel, B. C., Gunsalus, I. C., Wagner, G. C. & Kraut, J. *J. biol. Chem.* **260**, 16122–16130 (1985).
- Remington, S., Wiegand, G. & Huber, R. *J. molec. Biol.* **158**, 111–152 (1982).
- Brown, N. C., Eliasson, R., Reichard, P. & Thelander, L. *Eur. J. Biochem.* **9**, 512–518 (1969).
- Lynch, J. B., Juarez, G. C., Münch, E. & Que, L. Jr. *J. biol. Chem.* **264**, 8091–8096 (1989).
- Nordlund, P. et al. *FEBS Lett.* **258**, 251–254 (1989).
- Sjöberg, B. M., Karlsson, M. & Jönvall, H. *J. biol. Chem.* **262**, 9736–9743 (1987).
- Larsson, Å., Karlsson, M., Sahlin, M. & Sjöberg, B.-M. *J. biol. Chem.* **263**, 17780–17784 (1988).
- Sjöberg, B.-M., Loehr, T. M. & Sanders-Loehr, J. *Biochemistry* **21**, 96–102 (1982).
- Backes, G., Sahlin, M., Sjöberg, B.-M., Loehr, T. M. & Sanders-Loehr, J. *Biochemistry* **28**, 1923–1929 (1989).
- Sanders-Loehr, J. in *Iron Carriers and Iron Proteins* (ed. Loehr, T. M.) 375–466 (VCH Publishers, Deerfield Beach, 1989).
- Thelander, M. & Thelander, L. *EMBO J.* **8**, 2475–2479 (1989).
- McLachlan, A. D. *J. molec. Biol.* **128**, 49–79 (1979).
- Baker, E. N., Rumball, S. V. & Anderson, B. F. *Trends biochem. Sci.* 350–353 (1987).
- Volbeda, A. & Hol, W. G. J. *J. molec. Biol.* **206**, 531–546 (1989).
- Godzik, A. & Sander, C. *Protein Engng* **2**, 589–596 (1989).
- Stenkamp, R. E., Sieker, L. C., Jensen, L. H., McCallum, J. D. & Sanders-Loehr, J. *Proc. natn. Acad. Sci. U.S.A.* **82**, 713–716 (1985).
- Sherriff, S., Hendrickson, W. A. & Smith, J. L. *J. molec. Biol.* **197**, 273–296 (1987).
- Blum, M., Metcalf, P., Harrison, S. C. & Wiley, D. C. *J. appl. Crystallogr.* **20**, 235–242 (1987).
- Scarrows, R. C. et al. *J. Am. chem. Soc.* **108**, 6832–6834 (1986).
- Bunker, G. et al. *Biochemistry* **26**, 4708–4716 (1987).
- Bender, C. J. et al. *J. Am. chem. Soc.* **111**, 8076–8083 (1989).
- Sahlin, M. et al. *Biochemistry* **26**, 5541–5548 (1987).
- Sahlin, M., Gräslund, A., Petersson, L., Ehrenberg, A. & Sjöberg, B.-M. *Biochemistry* **28**, 2618–2625 (1989).
- Fontecave, M., Eliasson, R. & Reichard, P. *J. biol. Chem.* **264**, 9164–9170 (1989).
- Fontecave, M., Eliasson, R. & Reichard, P. *J. biol. Chem.* **262**, 12325–12331 (1987).
- Sahlin, M., Sjöberg, B.-M., Backes, G., Loehr, T. & Sanders-Loehr, J. *Biochem. biophys. Res. Commun.* **167**, 813–818 (1990).
- McClements, W. et al. *Virology* **162**, 270–273 (1988).
- Paradis, H., Gaudreau, P., Brazeau, P. & Langelier, Y. *J. biol. Chem.* **263**, 16045–16050 (1988).
- Terwillinger, T. C. & Eisenberg, D. *Acta crystallogr.* **A39**, 813–817 (1983).
- Wang, B. C. *Meth. Enzym.* **115**, 90–112 (1985).
- Bricogne, G. *Acta crystallogr.* **A32**, 832–847 (1976).
- Jones, T. A. & Thirup, S. *EMBO J.* **5**, 819–822 (1985).
- Jones, T. A. *Meth. Enzym.* **115**, 157–171 (1985).
- Jones, T. A., Bergdoll, M. & Kjeldgaard, M. in *Crystallographic and Modeling Methods in Molecular Design* (eds Bugg, C. & Ealick, S.) (Springer, New York, in the press).
- Brünger, T. A., Kuriyan, J. & Karplus, M. *Science* **235**, 458–460 (1987).
- Carlson, J., Fuchs, J. A. & Messing, J. *Proc. natn. Acad. Sci. U.S.A.* **81**, 4294–4297 (1984).
- Sjöberg, B.-M. et al. *EMBO J.* **5**, 2031–2036 (1986).
- Standart, N. M., Bray, S. J., George, E. L., Hunt, T. & Ruderman, J. V. *J. Cell Biol.* **100**, 1968–1976 (1985).
- Thelander, L. & Berg, P. *Molec. cell. Biol.* **6**, 3433–3442 (1986).
- Hurd, H. K., Roberts, C. V. & Roberts, J. W. *Molec. cell. Biol.* **7**, 3673–3677 (1987).
- Elledge, S. J. & Davis, R. W. *Molec. cell. Biol.* **7**, 2783–2793 (1987).
- Slabaugh, M., Roseman, N., Davis, R. & Mathews, C. *J. Virol.* **62**, 519–527 (1988).
- McLauchlan, J. & Clements, J. B. *EMBO J.* **2**, 1953–1961 (1983).
- Swain, M. & Galloway, D. *J. Virol.* **57**, 802–808 (1986).
- Davidson, J. A. & Scott, J. E. *J. gen. Virol.* **67**, 1759–1781 (1986).
- Gibson, T., Stockwell, P., Ginsburg, M. & Barel, B. *Nucleic Acids Res.* **12**, 5087–5099 (1986).

ACKNOWLEDGEMENTS. We thank Ulla Uhlin for her important contribution to early crystallization work and for drawing the schematic diagrams, and colleagues at the Biomedical Centre for suggestions and linguistic corrections in the manuscript. This work was supported by grants from the Swedish Natural Science Research Council, Swedish Medical Research Council (to B.-M.S.) and The Swedish Cancer Society (to B.-M.S.).

LETTERS TO NATURE

A 5.75-millisecond pulsar in the globular cluster 47 Tucanae

R. N. Manchester*, A. G. Lyne†, N. D'Amico‡, S. Johnston†, J. Lim§ & D. A. Kniffen||

* Australia Telescope National Facility, CSIRO, Epping, NSW, 2121, Australia

† University of Manchester, Nuffield Radio Astronomy Laboratories, Jodrell Bank, Macclesfield SK11 9DL, UK

‡ Istituto di Fisica dell'Università, via Archirafi 36, Palermo, Italy

§ School of Mathematics, Physics, Computing and Electronics, Macquarie University, North Ryde, NSW, 2113, Australia

|| Goddard Space Flight Center, Greenbelt, Maryland, 20771, USA

MILLISECOND pulsars are generally believed to be old pulsars that have been spun up ('recycled') as a result of accretion of matter from a companion in a low-mass X-ray binary system¹. As there is a high incidence of such systems in globular clusters², these are good places to search for millisecond pulsars; so far, ten globular-cluster pulsars have been detected unambiguously. Using the Parkes radiotelescope in Australia, we have found a pulsar with a period of 5.75 ms and a dispersion measure of 25 cm⁻³ pc in the direction of 47 Tucanae. Despite its probable origin as a member of a binary system, timing measurements show that the pulsar is now single. The observed dispersion measure is consistent with the pulsar lying outside the galactic electron layer and within 47 Tucanae; but it is very different from the value of 67 cm⁻³ pc for the pulsars that were reported recently^{3,4} as being in this globular cluster, and we suggest that the latter pulsars probably do not in fact lie within 47 Tucanae.

Over the past several months we have been searching for millisecond and short-period pulsars in globular clusters, super-

nova remnants and the galactic plane using the Parkes radio-telescope in the 20-cm and 50-cm wavelength bands. Receivers for both bands are dual-channel systems having field-effect-transistor preamplifiers which receive orthogonal linear polarizations. The 50-cm preamplifiers were cooled only after November 1989. Beamwidths are 14 arcmin and 32 arcmin for the 20-cm and 50-cm systems respectively and the system noises (equivalent flux density) on cold sky are 66 Jy, 130 Jy and 82 Jy for the 20-cm, ambient 50-cm and cooled 50-cm systems, respectively. Following down-conversion to an intermediate frequency, the signals are filtered in a 2 × 128 × 0.25-MHz multichannel filter for the 50-cm observations and in 2 × 64 × 5-MHz and 2 × 80 × 1-MHz filter systems for the 20-cm observations. After detection, low-pass filtering and summing of the orthogonal polarizations, the signals are sampled at 300-μs intervals by a multichannel one-bit digitizer system and recorded on magnetic tape.

Off-line analysis was carried out on a Convex C220 computer. For each of a number of candidate dispersion measures, the data were first dedispersed and then transformed to the spectral domain using a fast Fourier transform routine. The amplitude spectrum was normalized by subtraction of and division by a smoothed version of itself. Significant peaks were searched for in the normalized spectrum and after summing 2, 4, 8 and 16 harmonics. For the 50-cm data, 100 candidate dispersions between 0 and 309 cm⁻³ pc were searched.

The pulsar was first detected⁵ in analysis of 50-cm observations made in July 1989, and subsequently detected in analysis of 20-cm observations made in May and September 1988, and of 50-cm observations made in November–December 1989. Figure 1 shows normalized spectra for 50-cm data taken in an observation of 3,000-s duration on 16 July 1989, in the direction of 47 Tuc at dispersion measures of 16, 25 and 32 cm⁻³ pc. A strong signal at 173 Hz and three of its harmonics is evident in the

TABLE 1 Parameters of the 47 Tucanae pulsar

Right ascension (1950)	00 ^h 21 ^m 53 ^s (assumed)
Declination (1950)	-72°21'00" (assumed)
Barycentric period	5.756 780 090 ± 0.000 000 015 ms
Period derivative	(-4 ± 5) × 10 ⁻¹⁷
Epoch (Modified Julian Day)	47857.521
r.m.s. residual	84 µs
Dispersion measure	24.5 ± 0.5 cm ⁻³ pc
Half-power pulse width	0.9 ms
Mean flux density at 50 cm	3 mJy
Mean flux density at 20 cm	0.6 mJy

central dispersion spectrum. Figure 2 gives profiles obtained by folding data from the 128 individual frequency channels at the apparent period of 5.7567063 ms which show the characteristic square-law delay with frequency corresponding to the dispersion of 25 cm⁻³ pc. Modulation of the pulse spectrum, almost certainly due to interstellar scintillation, is evident. In the lower part of Fig. 2, the mean pulse profile resulting from summation of the individual channel profiles is given. The pulse is single and relatively broad with a full width at half-maximum of 0.9 ms or 15 per cent of the period. There is no evidence for an interpulse. In this observation the pulsar had a mean flux density of 7.5 mJy, although in several other observations in the same session the pulsar was undetected with a limit of ~1 mJy.

The pulsar was detected at the same dispersion measure in analyses of two of the three observations made in the direction of 47 Tuc with the 20-cm system and 5-MHz filters in May and September 1988. It was also detected in 10 of 12 observations made with the cooled 50-cm system in the period 28 November to 3 December 1989. Analysis of pulse arrival times from the last session gave the barycentric period parameters listed in Table 1. Quoted errors are twice the formal standard error derived from the least-squares fit. The pulsar position assumed is the nominal cluster centre; quoted errors do not include a contribution from uncertainty in this position. The small absolute value of the period derivative (which is insensitive to position errors of a few arcmin) shows that the pulsar is almost certainly single. As for other single millisecond pulsars, we presume that the erstwhile companion has been removed, either by evaporation⁶ or by collision in the dense core of the cluster^{7,8}. Flux densities are unweighted mean values for all observations;

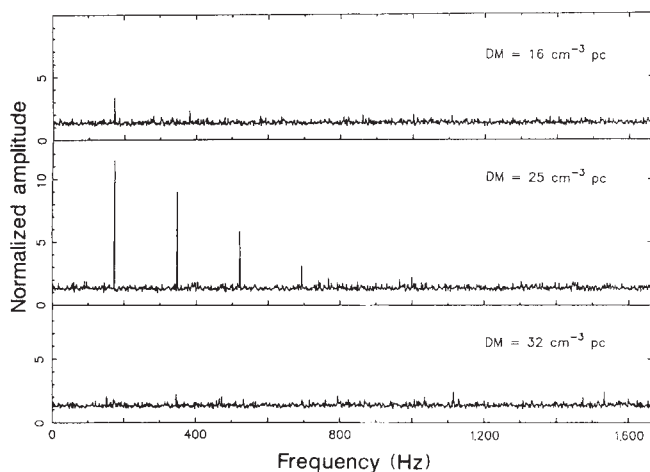


FIG. 1 Normalized modulation spectra for the 50-cm observation of 16 July 1989 in the direction of 47 Tuc, dedispersed for three different dispersion measures. For each spectrum, the 4×10^6 spectral amplitudes are binned into 1,024 bins and the highest amplitude in each bin is plotted. Spectral features resulting from the 5.75-ms pulse periodicity are clearly visible at the central dispersion measure of 25 cm⁻³ pc.

where the pulsar was not detected, three times the r.m.s. noise was assumed.

Is this pulsar in the globular cluster? Its detection at 20-cm wavelength shows that it is unlikely to lie more than 7 arcmin from the cluster centre, well within the bounds of the cluster. This by itself is quite convincing as the surface density of 'field' millisecond pulsars (those not in globular clusters) with flux densities >1 mJy at 50 cm, although not well determined, is of the order of 0.01 per square degree in the galactic plane⁹. At the galactic latitude of 47 Tuc (-45°) the density would be much less, so the probability of finding a field millisecond pulsar within the 20-cm beam is <0.0006. This already low probability would be reduced by another order of magnitude if the pulsar is identified with the variable continuum source located ~60 arcsec from the cluster centre which was observed at 843 MHz in Molonglo synthesis maps (A. J. Turtle, personal communication). Such an identification is suggested by the common variability and by the approximate equality of the maximum observed flux density of the continuum source, ~5 mJy, with the flux density of the pulsar (interpolated to 843 MHz) at the peak of a scintillation band.

For this pulsar, the 'perpendicular component' of the dispersion measure, $DM |\sin b|$, where b is the galactic latitude, is 17.7 cm⁻³ pc. If we exclude both the pulsar reported here and the two pulsars reported by Ables *et al.*^{3,4} to be in 47 Tuc, there are now ten pulsars known to be associated with seven different globular clusters. The mean value of $DM |\sin b|$ for these seven clusters is 19.8 cm⁻³ pc with an r.m.s. deviation of 6.4 cm⁻³ pc. This is consistent with most of those clusters lying outside the galactic electron layer. The estimated distance for 47 Tuc is 4.6 kpc (ref. 10) and hence it lies well outside the galactic electron layer. Therefore the $DM |\sin b|$ value for the pulsar reported

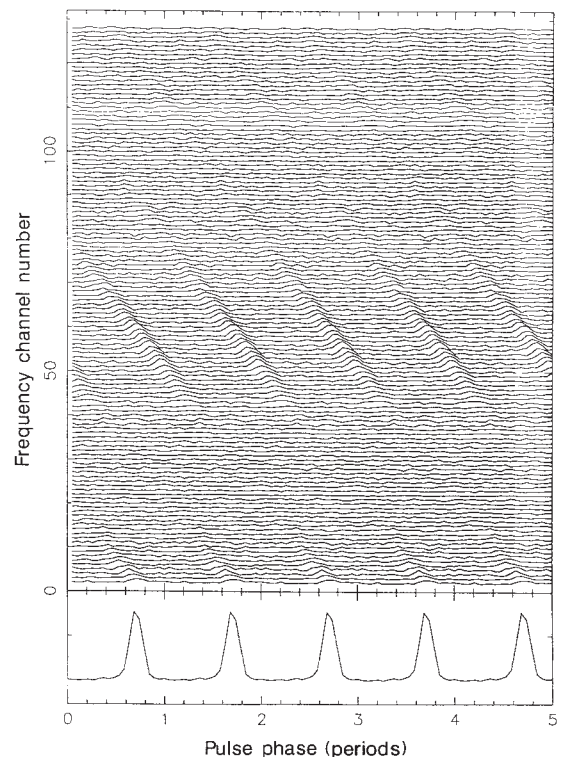


FIG. 2 Individual frequency channel data from the 50-cm observation of 16 July 1989 folded at the apparent pulsar period of 5.7567063 ms. Channel 1 corresponds to a received frequency of 624.125 MHz and channel 128 to 655.875 MHz. To show better the dispersive delay, the pulse profiles are repeated five times across the figure. The mean pulse profile obtained by summing across all frequency channels with compensation for the dispersive delay is shown at the bottom.

here is consistent with its being outside the galactic electron layer and in 47 Tuc.

As mentioned above, Ables *et al.*^{3,4} have reported the detection of two pulsars in 47 Tuc. One of these, PSR0021-72A, has a period of 4.479 ms and seems to be a member of a short-period binary system with an orbital period of 32 min. The other, PSR0021-72B, has a period of 6.127 ms and may be a member of a binary system with an orbital period of between 7 and 95 d. These pulsars have a dispersion measure of $67 \pm 2 \text{ cm}^{-3} \text{ pc}$, quite different to the value of $25 \text{ cm}^{-3} \text{ pc}$ for the pulsar reported here. We have searched our 50-cm data for these pulsars and have been unable to detect them. For the A pulsar we searched a total of 62 independent observations, each of duration ~ 15 min, over the range of period 4.4787-4.4791 ms and the range of period derivative $\pm 2 \times 10^{-10}$. These ranges more than cover the variations in apparent period and period derivative expected from the orbital motion of the pulsar and the Earth. Of the 62 observations, 40 were made with the more sensitive cooled-receiver system. No significant signal was detected at a dispersion of $67 \text{ cm}^{-3} \text{ pc}$ with a limit of 1.2 mJy for the cooled system. For a pulsar spectral index of -2 , this limit corresponds to 2.7 mJy at a frequency of 430 MHz which compares with a flux density at 430 MHz of $4 \pm 2 \text{ mJy}$ quoted by Ables *et al.*⁴. For the B pulsar we searched a total of 21 independent observations, most of which were of 33-min duration. All but five of these observations were made with the cooled receiver. Again we covered the ranges of period and period derivative expected from the pulsar and Earth orbital motion, namely 6.124-6.130 ms and $\pm 4 \times 10^{-11}$, and detected no significant signal. The limiting flux density is $\sim 0.9 \text{ mJy}$ at 50 cm, corresponding to 1.9 mJy at 430 MHz. No flux density value has been quoted by Ables *et al.*^{3,4} for the B pulsar, but it must be comparable to or greater than that of the A pulsar because that measurement was near their sensitivity limit.

The difference in dispersion measures of these pulsars is striking. It is very unlikely that this difference, $42 \text{ cm}^{-3} \text{ pc}$, could arise from ionized gas in the cluster. Excluding 47 Tuc, there are now three globular clusters (M5, M15 and NGC6624) where more than one pulsar has been detected. In each of these cases, the dispersion measures of the pulsars in the cluster are the same within errors of $3 \text{ cm}^{-3} \text{ pc}$ or less. These clusters all have dense cores with properties similar to those of 47 Tuc. Limits on H α emission from 47 Tuc (ref. 11) imply limits on the intracluster dispersion measure that are about an order of magnitude weaker. It is also very unlikely that the difference in dispersion measure could arise from different paths through the interstellar medium between here and 47 Tuc.

We conclude that the Ables *et al.* pulsars and the pulsar reported here cannot all be in 47 Tuc. The DM $|\sin b|$ value for the Ables *et al.*^{3,4} pulsars, $47.4 \text{ cm}^{-3} \text{ pc}$, is more than $16 \text{ cm}^{-3} \text{ pc}$ higher than that for any other cluster and is the highest known for any pulsar except those believed to be in the Magellanic Clouds. We think it is most likely that the 5.75-ms pulsar is in the cluster and hence that the Ables *et al.* pulsars are not. Position measurements, for example, from pulse timing measurements will help to resolve this question. $\square$

Received 23 February; accepted 19 April 1990.

- van den Heuvel, E. P. J. *J. Astrophys. Astr.* **5**, 209-233 (1984).
- Fabian, A. C., Pringle, J. E. & Rees, M. J. *Mon. Not. R. astr. Soc.* **172**, 15P-18P (1975).
- Ables, J. G. *et al.* *IAU Circ. No.* 4602 (1988).
- Ables, J. G. *et al.* *Nature* **342**, 158-161 (1989).
- Manchester, R. N. *et al.* *IAU Circ. No.* 4892 (1989).
- Ruderman, M., Shaham, J. & Tavani, M. *Astrophys. J.* **336**, 507-518 (1989).
- Romani, R. W., Kulkarni, S. R. & Blandford, R. D. *Nature* **329**, 309-310 (1987).
- Verbunt, F., van den Heuvel, E. P. J., van Paradijs, J. & Rappaport, S. A. *Nature* **329**, 312-314 (1987).
- Fruchter, A. S., Stinebring, D. R. & Taylor, J. H. *Nature* **333**, 237-239 (1988).
- Webbink, R. F. *Dynamics of Star Clusters*, IAU Symp. 113 (eds Goodman, J. & Hut, P.) 541-577 (Reidel, Dordrecht, 1985).
- Hesser, J. E. & Shaw, S. J. *Astrophys. J. Lett.* **217**, L143-L147 (1977).

ACKNOWLEDGEMENTS. We thank A. J. Turtle for making available to us details of his Molonglo observations of the 47 Tucanae region. N.D.A. acknowledges financial support from CNR-GIFCO and CNR Bilateral Projects. The Australia Telescope National Facility is operated in association with the Division of Radiophysics by CSIRO.

Unique series of increases in cosmic-ray intensity due to solar flares

T. Mathews & D. Venkatesan

Department of Physics and Astronomy, University of Calgary, Calgary, Alberta, Canada, T2N 1N4

THE second half of 1989 was the most prolific period of relativistic particle production by the Sun since continuous monitoring of cosmic-ray activity by neutron detectors was begun in 1957. Some of last year's events were among the largest bursts of particles observed throughout the last three solar cycles. Only when the particles released by the Sun during flares have energies greater than 0.5 gigaelectronvolt (GeV) are the secondary particles generated through interactions in the upper atmosphere able to reach ground level. Six different ground-level events were detected by the super neutron monitor in Calgary (geomagnetic latitude and longitude $51.08^\circ \text{ N } 245.91^\circ$, altitude 1,128 m, cut-off 1.07 GV) in the period July to October 1989. The intensity of energetic particle fluxes during these ground-level events is high enough to be a potential hazard, especially for passengers in aircraft at altitudes of 10 to 15 km.

Table 1 summarizes the main features of these six ground-level events (GLEs). The first two events were very small, typical of many GLEs observed during the last two solar cycles, and are not discussed further here. A seventh event on 15 November was observed at other stations, but not at Calgary.

The 29 September event was the largest in this set of GLEs. Indeed, with a peak intensity of $\sim 406\%$ above the pre-increase level, it is the largest event observed since the classic GLE on 23 February 1956. The event was also recorded by an underground muon telescope in New Mexico indicating that particles of energies at least up to 20 GeV were generated in this flare (D. W. Swinson, personal communication). Several unusual features are found when data from stations at Calgary, Inuvik, Goose Bay and Deep River in Canada and Oulu in Finland are

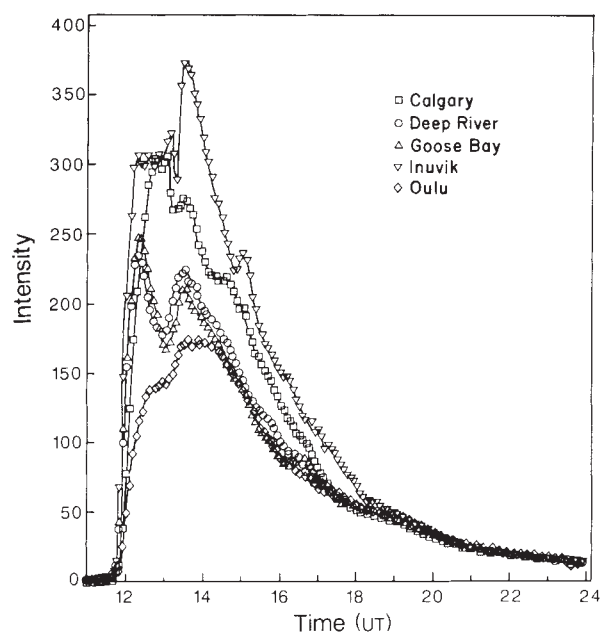


FIG. 1 GLE of 29 September 1989. Data (five-minute counting rates) from Calgary, Inuvik, Goose Bay, Deep River and Oulu. The background intensities have been subtracted.

TABLE 1 Features of the GLEs observed from Calgary

Date	$I_{\max}^*$ (%)	Onset time (UT)	Time of $I_{\max}$ (UT)
25 July 1989	2.9	9:10	09:45–09:50
16 August 1989	12.6	1:35	03:00–03:05
29 September 1989	405.6	11:50	12:55–13:00
19 October 1989	47.4	13:05	16:35–16:40
22 October 1989	30.1	18:00	18:55–19:00
	(68.9)	(18:00)	(18:90–18:10)
24 October 1989	106.7	18:25	20:15–20:20

* Maximum increase in intensity $I_{\max}$ is expressed as percentage of the average counting rate before the increases started. Background intensity has been subtracted. These values are five-minute counting rates.

TABLE 2 Observations at different stations on 29 September 1989

Station	Cut-off (GV)	Maximum intensity (%)	Time of maximum intensity (UT)
Inuvik (68.35° N, 226.27°)	0.17	321.6	13:05–13:10
Calgary* (51.08° N, 245.91°)	1.07	307.5	13:00–13:05
Deep River (46.10° N, 282.50°)	1.07	233.8	12:15–12:20
Goose Bay (53.33° N, 299.58°)	0.57	247.9	12:15–12:20
Oulu (65.00° N, 25.42°)	0.82	174.25	13:35–13:40

* Calgary is at mountain altitude (mean station pressure 883 mbar) and the intensities have been corrected to sea level using an absorption mean free path for solar-flare particles of 100 g cm^{-2} (refs 2, 3).

compared (Fig. 1). The onset of the event occurred at 11:50 UT at all stations, but the peak intensity was reached at different times (see Table 2). The profiles of the data are markedly different. For example, the Goose Bay and Deep River observations have double-peak profiles, and the highest intensity recorded at Calgary coincides with the time of minimum in the double-peak structure. The highest intensity, recorded at Inuvik, coincides with the time of the second maximum. To explain the differences in the intensity profiles at these stations, the asymptotic directions of approach of solar-flare particles arriv-

ing vertically at different locations must be examined¹. From these we expect the closest agreement between Goose Bay and Deep River and the largest difference between Oulu and Calgary or Inuvik, and this is what we observe.

The anisotropy is best illustrated by the data from Oulu and Calgary which have asymptotic directions about 160 degrees apart. Varying degrees of anisotropy are observed to exist for several hours (≥ 5) during this event. In previous GLEs such anisotropies were seen only during the rising phase of the events. These long lasting and varying anisotropies cannot be accounted for by a single explosive release of energetic particles. Delayed injection of particles into the interplanetary 'flux tubes' connecting Earth to the vicinity of the Sun may explain the observation. The largest increases in intensity were observed at stations with asymptotic directions in the general direction of the spiral interplanetary magnetic field towards the Sun.

Data for both the 19 and 22 October also show 'fine structure' in the intensity-time profiles. For the former event these features are barely noticeable, but for the latter event we observe a remarkable short-lived (< 20 min) increase during which the maximum intensity was 68.1% (Fig. 2). This occurred at 18:10 UT, when the asymptotic directions of 2–3 GeV particles arriving at Calgary were 30° east of the Earth–Sun line, that is, approximately at right angles to the interplanetary field line. This unusual spike was also observed by neutron monitors at the South Pole and at McMurdo in Antarctica (J. W. Bieber, personal communication) but not at Oulu. The arrival of particles from this direction has not been observed before in the initial stages in GLEs. Very unusual magnetic field structures must have been present in the interplanetary space near Earth at this time.

We can also compare these events by plotting the logarithm of the increase in intensity against time (Fig. 3). Solar-flare increases generally show an exponential decay with time during the late phase of the GLEs, the decay time constant being related to the details of the diffusion of solar-flare particles in interplanetary space. Here the slopes of the lines for different events are significantly different, leading us to conclude that rapid changes, on a timescale of the order of a week, were taking place in the interplanetary medium near Earth. A detailed analysis of these GLEs can only be attempted when data from several more ground-level stations and satellite-borne detectors become available.

These GLEs are the first to be observed during the current

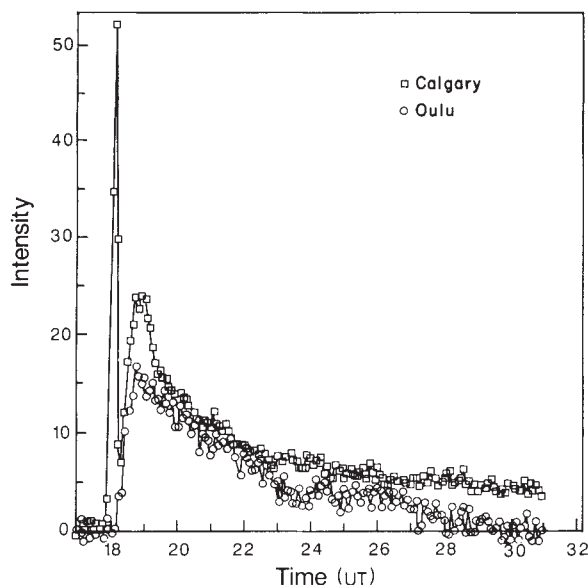


FIG. 2 GLE of 22 October 1989. Data (five-minute counting rates) from Calgary and Oulu.

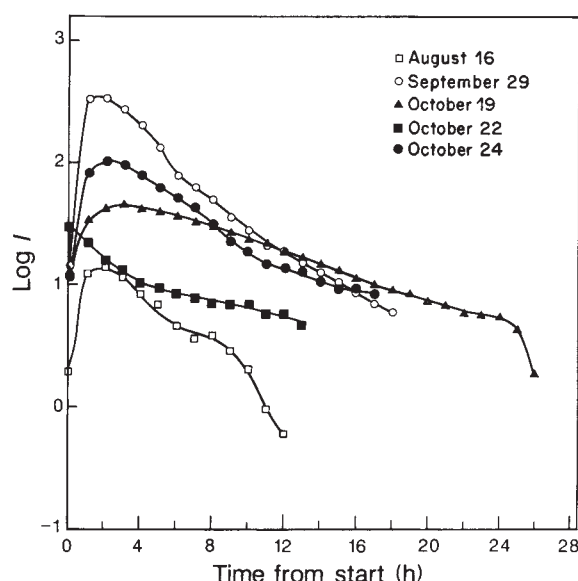


FIG. 3 Logarithm of intensity increases plotted against time for five GLEs.

cycle of solar activity, the exceptional nature of which is indicated by the very high mean monthly number of sunspots during this period (>180), the highest since 1959. The increase in intensity during the 29 September 1989 GLE was second only to the 23 February 1956 GLE which was also observed by neutron monitors near the Equator. The occurrence of six or seven events in such a short period makes the average particle radiation flux due to solar flares during the last six months of 1989 the highest ever found during a six-month period. $\square$

Received 20 February; accepted 26 April 1990.

1. Shea, M. A. & Smart, D. F. *Environ. Res. Pap.*, No. 510, AFCL-TR-75-0247 (Air Force Cambridge Research Laboratories, Hanscom, Mass., 1975).
2. McCracken, K. G. *J. geophys. Res.* **67**, 423-427 (1962).
3. Wilson, B. G., Mathews, T. & Johnson, R. H. *Phys. Rev. Lett.* **18**, 675-676 (1967).

ACKNOWLEDGEMENTS. We thank M. Bercovitch and M. Wilson for the data from other Canadian stations, H. Graumann for computational assistance, and P. Tanskanen for the use of his data. We acknowledge the support of the Natural Science and Research Council of Canada.

Superconductivity at 60 K in $\text{La}_{2-x}\text{Sr}_x\text{CaCu}_2\text{O}_6$: the simplest double-layer cuprate

R. J. Cava, B. Batlogg, R. B. van Dover, J. J. Krajewski, J. V. Waszczak, R. M. Fleming, W. F. Peck Jr, L. W. Rupp Jr, P. Marsh, A. C. W. P. James & L. F. Schneemeyer

AT&T Bell Laboratories, Murray Hill, New Jersey 07974, USA

STARTING with the pioneering work of Bednorz and Müller¹, many copper-oxide-based superconductors with high transition temperatures (T_c) have been discovered. All contain layers of copper-oxygen squares, pyramids or octahedra as their electronically active structural components^{2,3}. One structure type, first reported for $\text{La}_2\text{SrCu}_2\text{O}_6$ and $\text{La}_2\text{CaCu}_2\text{O}_6$ (ref. 4), has stood as an enigma since the beginning of high- T_c research. This crystal structure⁴⁻⁷ (Fig. 1) is the least complex of all the structures with the double layers of copper oxide pyramids common to the compounds with highest T_c , yet despite considerable effort, both published^{8,9} and unpublished, it has not until now been made superconducting. Here we report the successful synthesis and preliminary physical characterization of superconducting $(\text{La}, \text{Sr})_2\text{CaCu}_2\text{O}_6$. The highest transition temperature observed is 60 K at the composition $\text{La}_{1.6}\text{Sr}_{0.4}\text{CaCu}_2\text{O}_6$. This is a uniquely simple double-layer superconductor, which, like its single-layer analogue $(\text{La}, \text{Sr})_2\text{CuO}_4$, becomes superconducting through the introduction of carriers in an unambiguous manner—by straightforward atomic substitution without the intervention of charge reservoir layers with flexible valence states.

The starting materials for the synthesis were $\text{La}_2\text{C}_2\text{O}_4 \cdot 10\text{H}_2\text{O}$, $\text{Sr}(\text{NO}_3)_2$, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and CuO. After mixing according to the formula $\text{La}_{2-x}\text{Sr}_x\text{CaCu}_2\text{O}_{6-\delta}$, for $0.2 \leq x \leq 0.55$, powders were first dehydrated at 400 °C in air for 1 h. They were then slowly heated to 900 °C in flowing oxygen for several overnight cycles of calcining and mechanical grinding. After pressing into pellets, they were reacted for 1-3 days at 925 °C in flowing oxygen. The maximum strontium content obtained in solid solution was $x \approx 0.40$. Synthetic temperatures >950 °C clearly reduced the range of strontium substitution possible. Materials prepared in 1 atmosphere of oxygen can show superconducting transitions as high as 35 K. Using thermogravimetric analysis (heating in hydrogen gas to 1,000 °C) we found, however, that the oxygen content of an as-prepared single-phase $\text{La}_{1.6}\text{Sr}_{0.4}\text{CaCu}_2\text{O}_{6-\delta}$ sample was deficient, $\delta \approx 0.20$.

Materials were brought to near stoichiometric oxygen content ($\delta = 0$) by heating at moderately high oxygen pressures. Pellets were loosely wrapped in platinum foil and sealed in thick-walled quartz tubes containing enough oxygen (and liquefied by immersion of the tube in liquid nitrogen before sealing) to result in an oxygen pressure of 20 atm at 970 °C. Annealing times were 2 days at 970 °C, with subsequent gradual cooling and 5-h periods of annealing at 850, 750, 650 and 500 °C. Pellets had a brown coating where they were in contact with the platinum (a calcium platinum oxide) and this was completely removed before characterization. Samples that had a few per cent of a (non-superconducting) impurity after the 1-atm treatment were very pure (see below) for $x \leq 0.45$ after the high-pressure oxygen treatments, and reduction in hydrogen indicated near stoichiometric oxygen contents. Heating of $\text{La}_{1.6}\text{Sr}_{0.4}\text{CaCu}_2\text{O}_6$ to 800 °C in 1 atmosphere of oxygen resulted in a loss of ~ 0.1 oxygen per formula unit beginning at ~ 400 °C and completed by 600 °C. The oxygen lost was not reabsorbed on slow cooling in 1 atmosphere of oxygen.

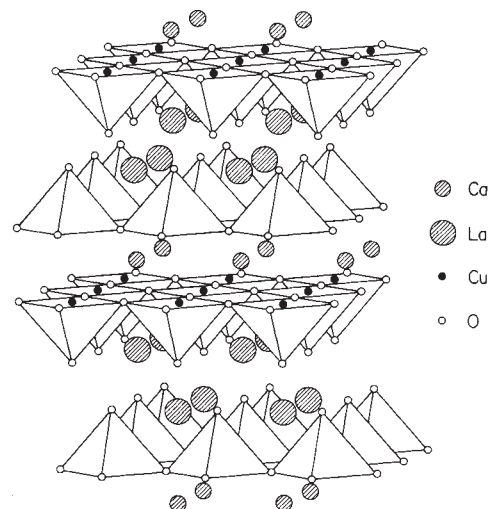


FIG. 1 The crystal structure of $\text{La}_2\text{CaCu}_2\text{O}_6$. It is related to La_2CuO_4 by replacing the layer of octahedra by a double layer of pyramids. The $\text{Ba}_2\text{YCu}_3\text{O}_7$ structure is derived from this one by adding CuO chains and shifting the pyramidal planes to align their apices.

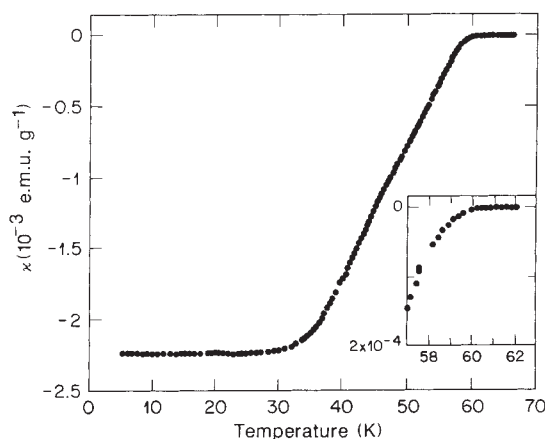


FIG. 2 Representative superconducting transition measured magnetically (d.c., 1.8-O_e field) on field cooling of $\text{La}_{1.6}\text{Sr}_{0.4}\text{CaCu}_2\text{O}_6$. Inset, detail of the temperature region near T_c .

The magnetic susceptibility of zero-field cooled samples of composition $x = 0.25\text{--}0.45$ is characteristic of full magnetic shielding in small a.c. and d.c. fields. To make a preliminary determination of the optimal superconducting strontium content, we measured the superconducting T_c and degree of flux expulsion on cooling in a d.c. magnetic field of 1.8 Oe; representative data are shown in Fig. 2. In the main panel the magnetic susceptibility in the vicinity of the superconducting transition of $\text{La}_{1.6}\text{Sr}_{0.4}\text{CaCu}_2\text{O}_6$ is shown. The amount of flux expelled is $\sim 20\%$ of that expected for an ideal compact diamagnet of the same weight and ideal density, indicating bulk superconductivity. The inset to Fig. 2 shows the detail in the temperature region near T_c , with the onset at 60 K.

Figure 3 shows the temperature-dependence of the resistivity between 4 and 300 K measured on a bar-shaped sample of $\text{La}_{1.6}\text{Sr}_{0.4}\text{CaCu}_2\text{O}_6$. The resistivity decreases with decreasing temperature, and approaches a constant value just before the

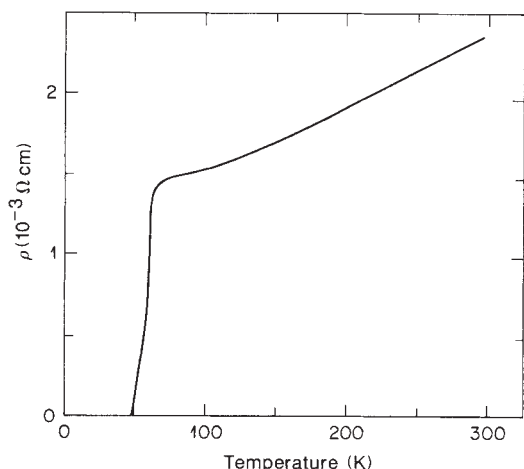


FIG. 3 Representative resistivity data for a polycrystalline sample of $\text{La}_{1.6}\text{Sr}_{0.4}\text{CaCu}_2\text{O}_6$.

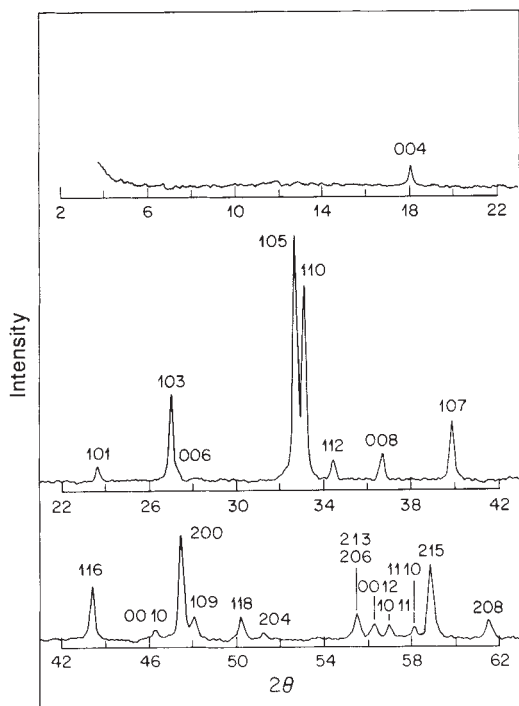


FIG. 4 Powder X-ray diffraction pattern of $\text{La}_{1.6}\text{Sr}_{0.4}\text{CaCu}_2\text{O}_6$. The unit-cell parameters of the tetragonal structure are $a = 3.82 \text{ \AA}$ and $c = 19.60 \text{ \AA}$.

onset of superconductivity. The superconducting transition of this sample is initially quite sharp, $\sim 2 \text{ K}$ wide, but there is a broad foot that extends the total transition width to $\sim 12 \text{ K}$. This feature is comparable to that seen in early samples of $\text{La}_{1.85}\text{Sr}_{0.15}\text{CuO}_4$ where it was attributed to weak coupling between grains¹⁰. The resistivity at room temperature, $\sim 2.3 \text{ m}\Omega \text{ cm}$, is a factor of four lower than that reported for $\text{La}_{1.9}\text{Ca}_{1.1}\text{Cu}_2\text{O}_6$ ($10 \text{ m}\Omega \text{ cm}$; ref. 8) at the same temperature. We expect that there is a significant grain-boundary resistivity in these materials and that improved ceramic processing or single crystals would show considerably lower resistivities and sharper resistive transitions.

The crystallographic unit cell of $\text{La}_2\text{CaCu}_2\text{O}_6$ is body-centred tetragonal with $a = 3.825$ and $c = 19.428 \text{ \AA}$ (refs 4–7). The symmetry and lattice centering are maintained in the solid solution, within the precision and sensitivity of the powder X-ray diffraction technique. For $\text{La}_{1.6}\text{Sr}_{0.4}\text{CaCu}_2\text{O}_6$, the cell dimensions, determined from the positions of several high-angle powder X-ray reflections, are $a = 3.82$ and $c = 19.60 \text{ \AA}$. The powder X-ray diffraction pattern for $\text{La}_{1.6}\text{Sr}_{0.4}\text{CaCu}_2\text{O}_6$ is presented in Fig. 4. There are no impurity peaks present above the detection limit of $\sim 1\%$. In the following, an even more stringent test of the purity is described.

The superconducting transition temperatures of the well-known superconductors $\text{La}_{2-x}\text{M}_x\text{CuO}_4$ do not exceed 36–39 K under one atmosphere or high-pressure synthetic conditions^{10–12}. Our observed T_c s, near 60 K, therefore exclude those K_2NiF_4 -structure compounds, even if present as low-level impurities, from being the source of the superconductivity in our materials. Nevertheless, we have prepared single-phase $\text{La}_{1.85}\text{Sr}_{0.15}\text{CuO}_4$ and $\text{La}_{1.85}\text{Ca}_{0.15}\text{CuO}_4$ by the synthetic procedures used here and have found their superconducting T_c s to be $< 38 \text{ K}$, as expected. Further, we have looked in detail at the region of the powder X-ray diffraction pattern ($30\text{--}34^\circ 2\theta$ for $\text{Cu K}\alpha$ radiation) of the $\text{La}_{1.6}\text{Sr}_{0.4}\text{CaCu}_2\text{O}_6$ samples where the main diffraction peaks of the $\text{La}_{2-x}\text{M}_x\text{CuO}_4$ superconductors or other perovskite-like phases are expected to occur. There is a complete absence of impurity diffraction peaks in that area down to the detection limit of approximately two standard deviations of the background intensity, 50 counts, when the main diffraction peak from $\text{La}_{1.6}\text{Sr}_{0.4}\text{CaCu}_2\text{O}_6$ contains in excess of 95,000 counts. This very low detection limit means that other perovskite-related phases could not be present in excess of 5×10^{-4} of the sample, an amount many orders of magnitude too low to account for the observed diamagnetism.

The T_c of $\text{La}_{2-x}\text{Sr}_x\text{CaCu}_2\text{O}_6$, 60 K, is what one might expect for a double-layer CuO_2 network embedded in a highly ionic medium, that is, the La–Sr–Ca–O framework shows no flexibility in the charge state of its components. The double-layer materials that have higher T_c s commonly involve interleaving with charge reservoir layers, which are considerably more covalent, allowing more flexibility in the internal redistribution of charge, through the apical oxygen of the CuO_5 pyramids. Our preliminary determination of optimal strontium content for $(\text{La}, \text{Sr})_2\text{CaCu}_2\text{O}_6$ results in a hole concentration of 0.20 per Cu, only slightly higher than the 0.175 per Cu optimal for the single layer $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$. If this is confirmed, it implies either a very large change in T_c with a small increase in hole concentration, or it implies that the universal curves of T_c as a function of hole concentration, which ignore factors such as the number of copper planes and the strength of their electronic coupling, or the number of apical oxygen copper–oxygen coordination geometries (for example, the number of apical oxygens), cannot tell the whole story. □

Received 21 May; accepted 30 May 1990.

1. Bednorz, J. G. & Müller, K. A. *Z. Phys.* **B64**, 189–193 (1986).
2. Sleight, A. W. *Science* **242**, 1519–1527 (1988).
3. Cava, R. J. *Science* **247**, 656–662 (1990).
4. Nguyen, N., Er-Rakho, L., Michel, C., Choisinet, J. & Raveau, B. *Mater. Res. Bull.* **15**, 891–897 (1980).

5. Santoro, A., Beech, F. & Cava, R. J. *High T_c Symposium, Materials Research Society Proceedings Series Vol. 169* (in the press).
6. Izumi, F., Takayama-Muromachi, E., Nakai, Y. & Asano, H. *Physica C* **157**, 89 (1989).
7. Caignaert, V., Nguyen, N. & Raveau, B. *Mater. Res. Bull.* **25**, 199–204 (1990).
8. Tamagai, T. & Iye, Y. *Physica C* **159**, 181–187 (1989).
9. Torrance, J. B., Tokura, Y., Nazzal, A. & Parkins, S. S. P. *Phys. Rev. Lett.* **60**, 542–545 (1988).
10. Cava, R. J., van Dover, R. B., Batlogg, B. & Reitman, E. A. *Phys. Rev. Lett.* **58**, 408–410 (1987).
11. Tarascon, J. M., Greene, L. H., McKinnon, W. R., Hull, G. W. & Geballe, T. H. *Science* **235**, 1373–1375 (1987).
12. Torrance, J. B. et al. *Phys. Rev. Lett.* **61**, 1127–1131 (1988).

Microstructure of diffusive boundary layers and the oxygen uptake of the sea floor

Jens K. Gundersen & Bo Barker Jørgensen

Department of Ecology and Genetics, University of Aarhus, Ny Munkegade, DK-8000 Aarhus C, Denmark

THE diffusive boundary layer (DBL) is a thin (≤ 1 mm) film of water that covers the sea floor, and through which molecular diffusion is the dominant transport mechanism for dissolved material. The diffusive fluxes are a measure of the rate of remineralization of organic matter in the sea bed, and of the dissolution or precipitation of minerals such as carbonates or metal oxides. Here we report detailed *in situ* analyses of chemical microgradients within the DBL, using a microelectrode profiling instrument with a spatial resolution of 25–50 μm . Over a Danish coastal sediment at 15 m water depth, the DBL was 0.5–0.7 mm thick and showed both stochastic fluctuations of oxygen distribution owing to boundary-layer turbulence and harmonic oscillations resulting from surface waves. A three-dimensional mapping of the DBL and the corresponding sediment surface showed that the DBL

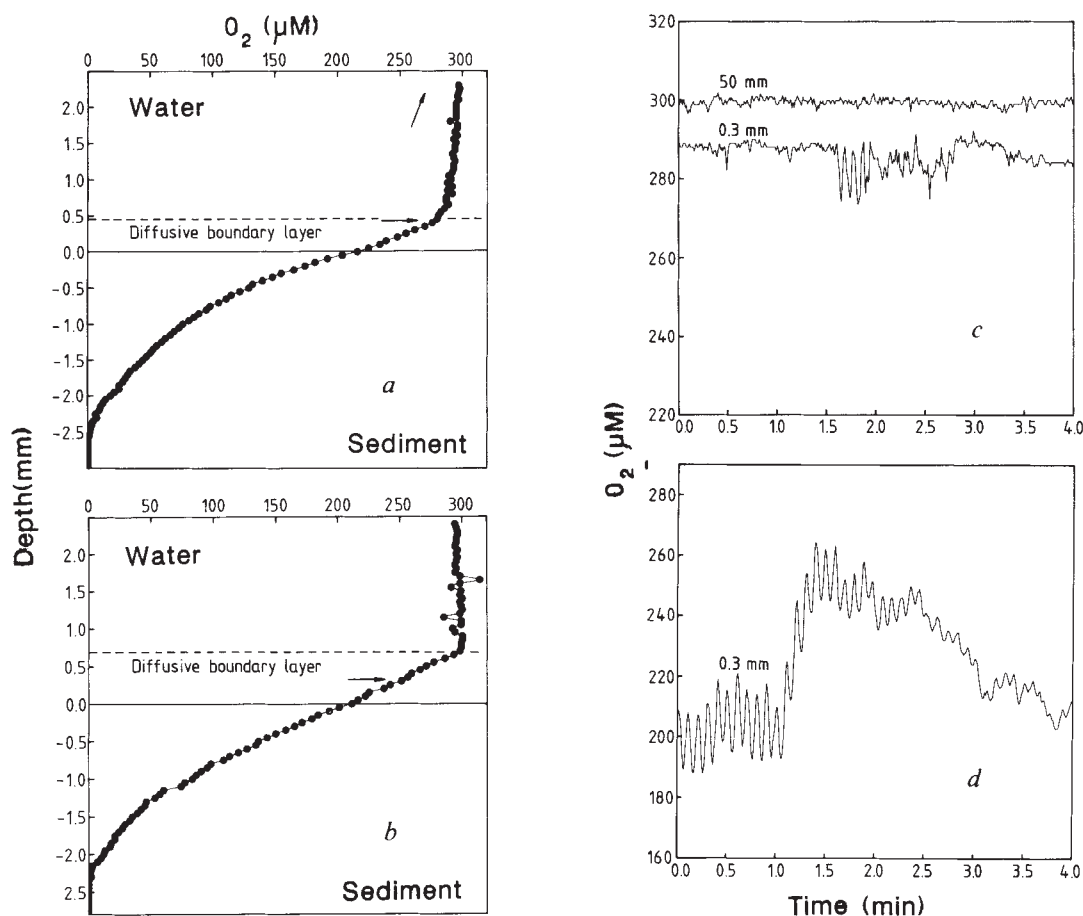
was spatially well defined and followed surface contours, but smoothed out sediment microtopographic features smaller than 100 μm . The three-dimensional oxygen diffusive flux across the sediment/water interface was about 2.5 times higher than that calculated from a simple one-dimensional diffusion model. These results indicate that benthic oxygen consumption and other fluxes can be studied by direct measurement of DBL microgradients at the undisturbed sea floor.

The vertical transport of dissolved material by eddy diffusion is strongly reduced at the transition from the turbulent water flow above the sea floor to the linear flow near the sediment/water interface. The eddy diffusion coefficient K decreases as $z^{3.38}$ (ref. 1) (where, z is height above the sediment) in the centimetre-thick viscous sublayer next to the sediment surface, whereas the molecular diffusion coefficient D is independent of flow and thus independent of z . The upper boundary of the DBL is found where K becomes smaller than D . The transition from the uniform distribution of dissolved material in the turbulent water to the linear diffusion gradients of the DBL occurs 0.2–1 mm above the sediment surface^{2–5}.

The DBL constitutes an important diffusion barrier to many dissolved species and affects the exchange of oxygen, nutrients and metal ions at the sea floor^{3,6,7}. The DBL thus has a significant role for benthic respiration, especially in shelf sediments with high oxygen uptake rates. The DBL may also control the dissolution of calcite or the accretion of ferromanganese nodules in the deep sea. But the DBL of the sea floor has not been measured directly in most earlier studies — its effective thickness has been calculated indirectly from modelling of, for example, dissolution rates of alabaster plates or from rates of sediment–water oxygen exchange^{6–8}.

Oxygen microelectrodes are excellent tools for boundary-layer studies because of their high spatial and temporal resolution. We used Clark-type microelectrodes, constructed in our labora-

FIG. 1 *a* and *b*, Oxygen distribution at the sediment/water interface in Aarhus Bay at 15 m water depth. *c*, After the measurement of profile in *a*, the stability and fluctuations of O_2 concentration were analysed at 50 mm above the sediment and in the DBL (marked by arrows in *a*). *d*, Harmonic oscillations of O_2 concentration owing to wave action were demonstrated in the DBL of *b*. Electrodes were calibrated linearly between the O_2 concentration in the bulk water (determined by Winkler titration) and the depth-independent zero current below the O_2 zone. Oxygen signals were averaged over 0.3 s and measurements for depth distributions were taken at 4-s intervals.



tory, with a sensing-tip diameter of $<10\text{ }\mu\text{m}$, a 90%-response time of 1 s, and a velocity sensitivity of $<1\text{--}2\%$ ^{9,10}. The microelectrodes were mounted on a benthic *in situ* profiling instrument¹¹. The supporting tripod was lowered to the sea floor by wire and cable, which allowed on-line data collection on board the ship. The instrument can also be operated to water depths of 6,000 m as a preprogrammed free vehicle with 128 kbytes of data storage capacity. Remote observations of sediment and electrode operations were made with an attached underwater video camera. The tripod was allowed to stabilize on the sea floor for an hour and oxygen microgradients were measured at depth intervals of $50\text{ }\mu\text{m}$. This spatial resolution is ten times higher than that obtained during earlier *in situ* measurements^{5,12}. Water flow at 5 cm above the sediment was determined simultaneously by analysing video tape of the transport of suspended particles.

Figure 1 shows two examples of benthic oxygen microgradients in sediments of sandy mud along the sheltered Danish coast. Oxygen was consumed within the top 2.2–2.5 mm of the

sediment by intensive microbial respiration. The DBL thicknesses of 0.48 mm (a) and 0.68 mm (b) were determined from the sharp transition (upper DBL boundary) between the homogeneous oxygen distribution in the turbulent water column and the linear gradient in the DBL. The oxygen concentration gradients, dC/dz , showed a small but sharp increase of 35% (a) and 21% (b) across the sediment/water interface (lower DBL boundary) owing to the impeded diffusion in the sediment relative to that in the water^{13,14}. The break in the O_2 gradient was used as a precise indication of the vertical position of the chemical profile relative to the sediment surface. Such an accurate positioning is crucial for the determination of DBL thickness. At the present 25–50 μm resolution, however, the sediment/water interface was often not well defined and the O_2 gradient did not show a distinct break in such profiles. An independent approach should therefore be developed to experimentally distinguish the interface.

When the O_2 gradient in the DBL is determined at high resolution, the diffusive flux J of O_2 across the sediment/water

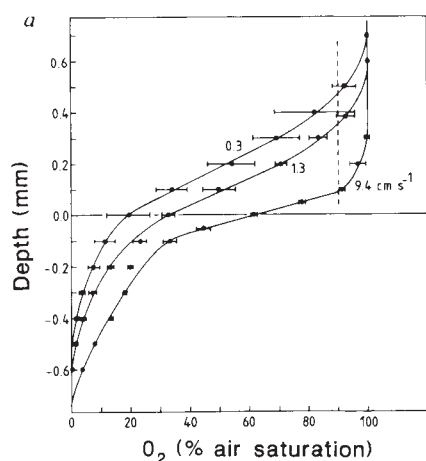
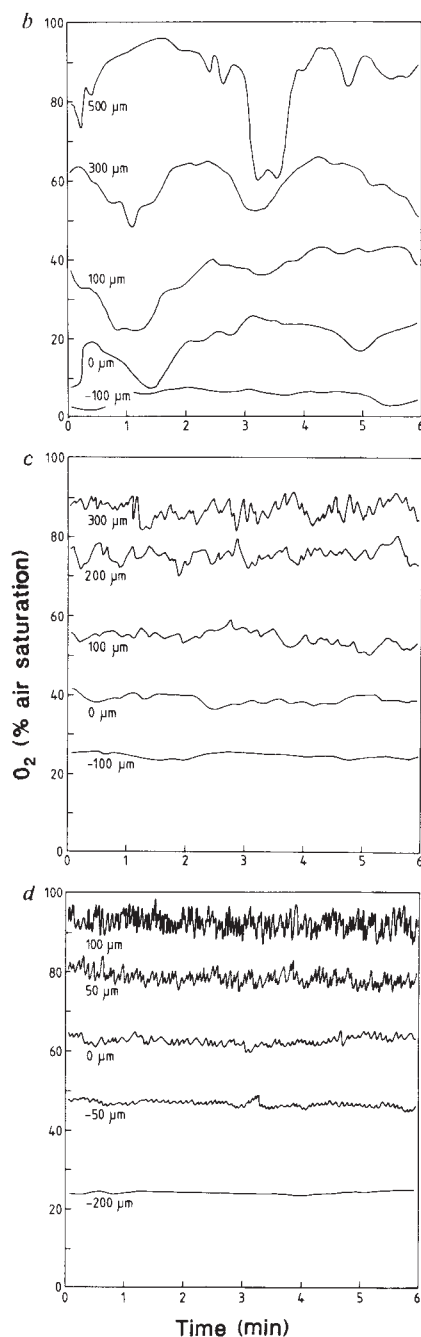


FIG. 2 Oxygen distribution and fluctuations at the sediment/water interface at different velocities of water flow. An undisturbed core of fine-grained sediment from a Danish coastal lagoon was placed in an aquarium with the sediment surface positioned flush with a smooth acrylic plate (B.B.J. and D. J. Des Marais, manuscript submitted). A regulated, horizontal flow of sea water over the sediment established a boundary layer, which was hydrodynamically not fully developed, but in which the DBL was of similar thickness as *in situ* under similar flow velocities. a, Mean O_2 distributions (with standard deviations) were based on four profiles measured for each of three flow velocities, 0.3, 1.3 and 9.4 cm s^{-1} . b, c, d, Temporal fluctuations of O_2 concentration at flow velocities 0.3, 1.3 and 9.4 cm s^{-1} respectively, measured with a microelectrode positioned at decreasing depths relative to the sediment surface. At a flow velocity of 1.3 cm s^{-1} , the frequency of fluctuations was 3.8, 5.7 and 8.2 min^{-1} at 0.1, 0.2 and 0.3 mm, respectively; at a flow velocity of 9.4 cm s^{-1} , f was 6.1, 14.8 and 18.2 min^{-1} at 0.0, 0.05 and 0.10 mm.



interface can be calculated from $J_{\text{water}} = D(dC/dz)_{\text{water}}$ (ref. 15). This determination is simpler than a flux calculation just below the sediment surface where the O_2 gradient may be easier to measure but where determination of sediment diffusion coefficient and porosity at high spatial resolution is required¹². The diffusive flux of O_2 into the sediment of Aarhus Bay was 19.8 and 15.1 $\text{mmol m}^{-2} \text{d}^{-1}$ as calculated from the DBL gradients of Fig. 1a and b respectively ($D = 1.49 \times 10^{-5} \text{ cm}^2 \text{s}^{-1}$ at 8°C)¹⁶. A concurrent seasonal study of benthic respiration was carried out with sediment cores retrieved from the same position in Aarhus Bay. The diffusive flux of O_2 across the sediment/water interface calculated from O_2 microgradients accounted for 69% of the total O_2 uptake (H. Rasmussen and B.B.J., manuscript in preparation).

The oxygen concentration at 50 mm above the sediment was $299.7 \pm 0.9 \mu\text{M}$ (± 1 s.d.), whereas in the DBL fluctuations were induced by turbulence in the water above (Fig. 1c). While the profile in Fig. 1b was being recorded, waves on the water surface 15 m above caused harmonic oscillations of the bottom current in the range $0\text{--}12 \text{ cm s}^{-1}$ (mean $= 6 \text{ cm s}^{-1}$) at a frequency of 10 min^{-1} . These oscillations were transmitted to the DBL where this 'wave pumping' may enhance the vertical oxygen transfer as do other low-frequency velocity fluctuations¹⁷ (Fig. 1d).

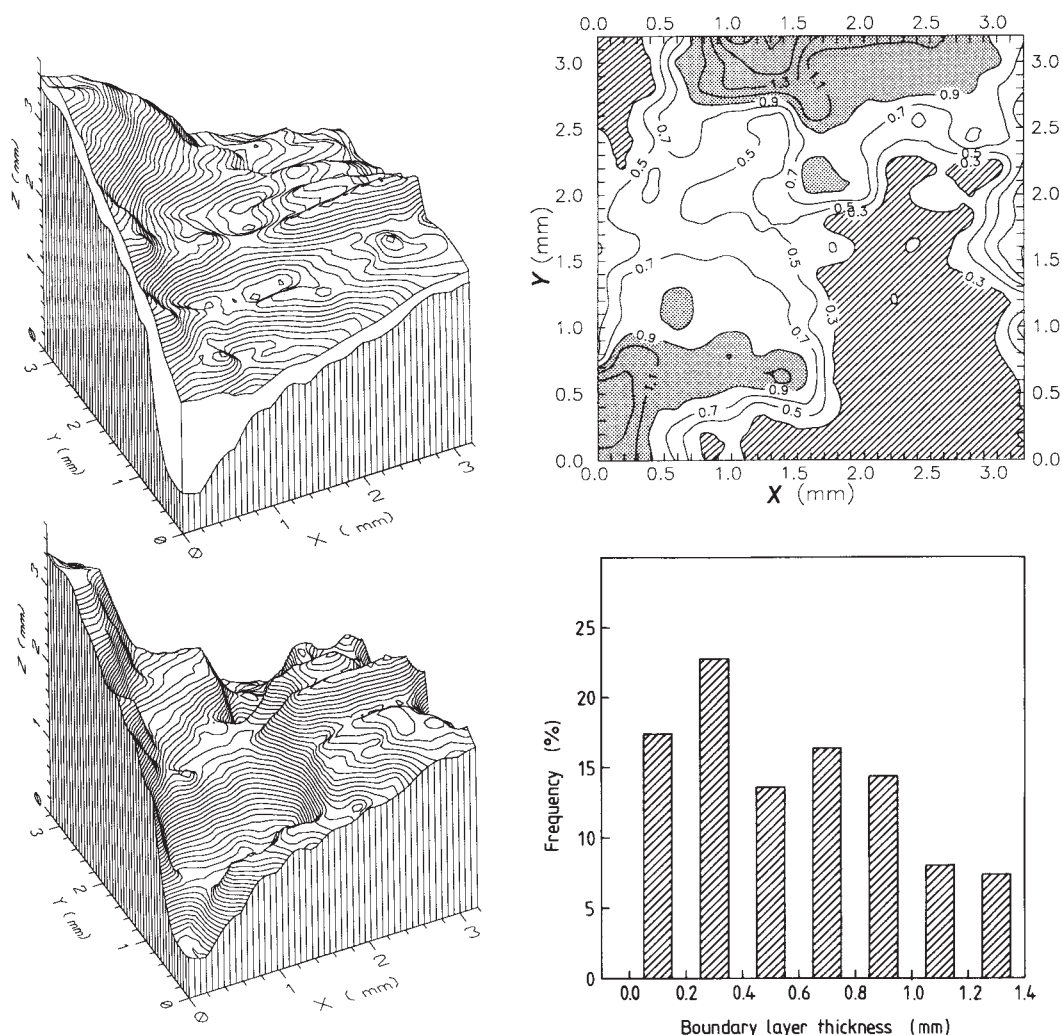
The analysis of time series of O_2 fluctuations from *in situ* measurements in the DBL provides a new approach to understand the fluid dynamics of the benthic boundary layer. The 'noise' in the oxygen measurements reflects stochastic fluctuations of the DBL owing to numerous eddies which approach the sediment surface from the bulk of the flowing sea water and hit the viscous and diffusive sublayers¹⁸. We studied the instabil-

ity of the DBL further in the laboratory where sediment cores were exposed to different flow velocities (Fig. 2). The experimental increase of flow velocity from 0.3 to 9.4 cm s^{-1} had several effects: (1) the thickness of the DBL decreased from 0.48 to 0.13 mm ; (2) the oxygen gradient and thus the diffusive flux of oxygen increased twofold; (3) the oxygen concentration at the sediment surface increased from 19% to 62% of the air-saturation value; (4) the stability of the oxygen gradient, and thus the DBL, increased.

The frequency f of the stochastic fluctuations in the DBL is theoretically a function of distance z from the surface, of the friction velocity U_* and of the kinematic viscosity, ν : $f = 0.07 z U_*^2 / 243 \nu \text{ s}^{-1}$ (ref. 18). We found that the frequency did indeed increase strongly with both z and flow velocity (Fig. 2). Figure 2 also shows that the transition from fluctuating to stable oxygen concentration is not a reproducible indicator of the sediment/water interface because the position of the transition varies with flow velocity. At high flow velocity the oxygen concentration showed eddy-induced fluctuations even at 0.05 mm below the sediment surface.

On the scale of the DBL or of the microelectrode tip, the sediment surface is a rugged landscape of 'mountains' and 'valleys' which can be mapped and described using topographic methods. Figure 3 shows an example of the topography of the sediment surface and of the corresponding upper boundary of the DBL over an area of 10 mm^2 . At each point, the upper boundary of the DBL was defined where the oxygen concentration had fallen to 10% below the bulk level, and the sediment surface was determined as the position where the microelectrode first touched a solid particle⁴. Like a thin blanket of water, the

FIG. 3 Topographic maps of the sediment surface and the diffusive-boundary-layer surface over a 10-mm^2 sediment area. The sediment was covered by a 2-cm-thick water layer flowing at a mean velocity of 0.6 cm s^{-1} in the negative direction along the Y-axis. The three-dimensional graphs were constructed from a grid of 17×17 measuring points at 0.05-mm depth resolution and 0.2-mm horizontal resolution. Lower left, the sediment relief was mapped relative to an arbitrary base level. Upper left, the DBL surface mapped on the same scale as the sediment surface. Upper right, topographic map of DBL thickness with the thinnest regions hatched ($< 0.3 \text{ mm}$) and the thickest regions shaded ($> 0.9 \text{ mm}$); numbers on contours are DBL thicknesses in mm. Lower right, histogram of DBL-thickness frequency distribution for the same surface.



DBL followed the topography of the sediment surface being thinner over the 'mountains' and thicker over the 'valleys'. The DBL thickness varied from <0.2 to >1.5 mm with a mean thickness of 0.59 mm (Fig. 3).

The DBL topography shows the importance of describing the three-dimensional diffusion field of O_2 rather than just the vertical gradients. At the spatial resolution of Fig. 3, the rugged sediment surface had an effective area 88% larger than a flat surface. The DBL surface was 35% larger than a flat surface. Based on simple geometric considerations (a double cosine correction for the mean slopes of sediment and DBL surfaces) (B.B.J. and D. J. Des Marais, manuscript submitted), we have calculated that the three-dimensional diffusion flux of oxygen across the sediment/water interface is 2.5 times the flux calculated from a one-dimensional model based on the mean vertical DBL gradient. This explains, in part, why direct measurements of sediment O_2 uptake yield higher fluxes than those calculated from O_2 microgradients.

Systematic use of this experimental technique can lead to a better quantitative understanding of the relation between surface relief, flow velocity, DBL topography and diffusive flux. In combination with the microelectrode analyses of stochastic fluctuations such data can be used to determine directly the mechanisms of mass transfer through the DBL. The determination of DBL distribution and stability from oxygen microgradients can be used also to understand its role in the sediment-water exchange of other solutes of ecological or geochemical

importance. With the *in situ* microprofiling instrument it is now possible to study the diffusive boundary layer under natural, undisturbed conditions of the sea floor at high spatial and temporal resolution. □

Received 11 January; accepted 16 April 1990.

1. Shaw, D. A. & Hanratty, T. J. *Am. Inst. chem. Eng. J.* **23**, 28–37 (1977).
2. Boudreau, B. P. & Guinasso, N. L. Jr in *The Dynamic Environment of the Ocean Floor* (eds Fanning, K. A. & Manheim, F. T.) 111–145 (Lexington, Massachusetts, 1982).
3. Santchi, P. H., Bower, P., Nyffeler, U. P., Azevedo, A. & Broecker, W. S. *Limnol. Oceanogr.* **28**, 899–912 (1983).
4. Jørgensen, B. B. & Revsbech, N. P. *Limnol. Oceanogr.* **30**, 111–122 (1985).
5. Archer, D., Emerson, S. & Smith, C. R. *Nature* **340**, 623–626 (1989).
6. Boudreau, B. P. *Am. J. Sci.* **288**, 777–797 (1988).
7. Hall, P. O. J. *et al. Limnol. Oceanogr.* **34**, 734–746 (1989).
8. Schink, D. R. & Guinasso, N. L. Jr in *The Fate of Fossil Fuel CO_2 in the Oceans* (eds Andersen, N. R. & Malahoff, A.) 375–399 (Plenum, New York, 1977).
9. Revsbech, N. P. *Limnol. Oceanogr.* **34**, 474–478 (1989).
10. Revsbech, N. P. & Jørgensen, B. B. *Adv. microbiol. Ecol.* **9**, 293–352 (1986).
11. Reimers, C. E. *Deep Sea Res.* **34**, 2019–2035 (1987).
12. Reimers, C. E., Fischer, K. M., Merewether, R., Smith, K. L. Jr. & Jahnke, R. A. *Nature* **320**, 741–744 (1986).
13. Revsbech, N. P. *J. Microbiol. Meth.* **9**, 111–122 (1989).
14. Swerts, J.-P. R. A., St Louis, V. & Cappenberg, T. E. *Freshwater Biol.* **21**, 401–409 (1989).
15. Crank, J. *The Mathematics of Diffusion* (Clarendon, Oxford, 1975).
16. Broecker, W. S. & Peng, T.-H. *Tellus* **26**, 21–35 (1974).
17. Campbell, J. A. & Hanratty, T. J. *Am. Inst. chem. Eng. J.* **29**, 215–221 (1983).
18. Dworak, R. & Wendt, H. *Ber. Bunsenges. phys. Chem.* **81**, 864–869.

ACKNOWLEDGEMENTS. We are grateful to E. Larsen and F. Hansen for their participation in the construction of an *in situ* microprofiling instrument, M. B. Christiansen, C. E. Reimers and D. Archer for advice during the development of the instrument, and B. Boudreau for discussions of boundary-layer theory. This work was supported by the Danish Ministry of the Environment and the Danish Natural Science Research Council.

Diffusion of rare-earth elements in fish teeth from deep-sea sediments

Kazuhiro Toyoda & Masayasu Tokonami

Mineralogical Institute, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

THE distribution of rare-earth elements (REEs) in biogenic phosphate phases in sediments (such as the remains of fish teeth) has been proposed as an indicator of the depositional environment in the water column, recording for example palaeo-redox conditions¹ and isotope geochemical^{2–5} and hydrothermal^{6,7} processes. Enrichment of these phases in REEs takes place only during early diagenesis^{8,9} for which reason the REE signal has often been assumed to represent the unfractionated record of REE distribution in the water column overlying the sediment surface^{1,4,5,10}. Here we report on studies of REE zoning in fish teeth from Pacific sediments, from which we extract a REE diffusion coefficient of $\sim 70 \text{ mm}^2 \text{ Myr}^{-1}$. This value suggests that diffusion of REEs from sea water into the fish teeth before burial is too slow to produce the enrichments observed. Instead, the REE distributions must derive from the surrounding sediment pore fluids. The use of these distributions as palaeoceanographic indicators is therefore called into question.

We chose fish-debris samples from the 30–40-cm core depth from piston-core sample KH 70-2-9-3 ($17^\circ 06' \text{ N}$, $146^\circ 12' \text{ W}$) collected in the central Pacific during the cruise of the AV *Hakuhou Maru*. Palaeomagnetic studies¹¹ show that the average sedimentation rate at site KH 70-2-9-3 is $\sim 7 \text{ mm kyr}^{-1}$, given a sample age of $\sim 50 \text{ kyr}$. The sediment sample of KH 70-2-9-3 is a red clay that has a large negative Ce anomaly and a high content of REEs¹².

Fish-debris grains were hand-picked and cleaned with distilled water, and were classified as either fish teeth or massive fragments. The grains of fish teeth (except for the base) are almost completely covered by enamel and the grains are composed mostly of dentine. The trace-element content of four

fish-debris samples (grain sizes range from 0.5 mm to 0.2 mm) was determined by instrumental neutron activation analysis (INAA)¹³. Shale-normalized REE patterns of the fish debris (Fig. 1) indicates that it has ~ 100 times the REE abundances of shale¹⁴ with large negative Ce anomalies and heavy REE enrichment. The similarity of REE patterns to the literature values for fish debris⁸ and sea water^{15,16} shows that our samples are typical REE-enriched biogenic apatite.

The distribution of REEs in several grains of fish debris were determined by electron-probe microanalysis (EPMA) (JEOL JMC-A 733 Mk-II)^{17,18}. A typical La distribution is shown in Fig. 2. We found chemical zoning of REEs parallel to the lineation of fish teeth—La, Sm and Yb are concentrated at the

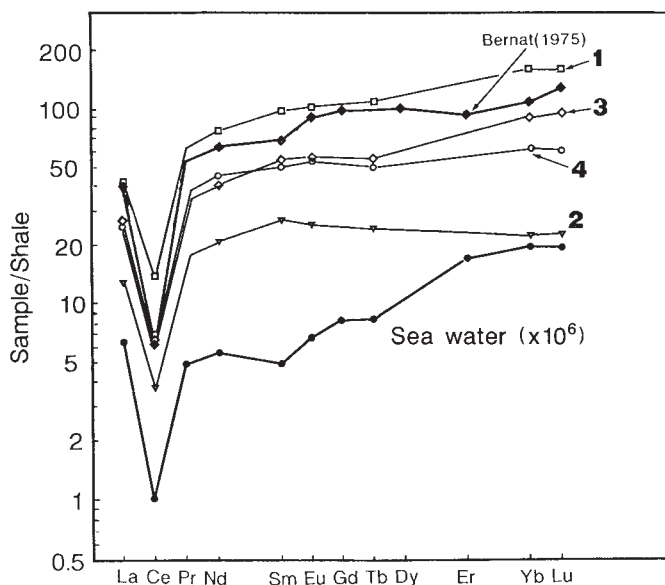


FIG. 1 Comparison of shale-normalized REE patterns¹⁴ of our four fish-debris samples (open symbols) with those of fish debris from Pacific deep-sea sediment⁸ (◆) and sea water¹⁵ (●).

base and depleted at the tip. The REE distribution of other fish tooth samples are similar.

Figure 2 also shows the map of calcium and phosphorus content, the distribution of which should reflect the structure of teeth. High levels of Ca and P are observed in the grain margin. We determined that the $\text{Ca}_2\text{P}_2\text{O}_7$ content in the rim of the grain is 80–90% (enamel), whereas that in the centre is 65–80% (dentine)^{19,20}. The remainder is considered to be composed of organic matter, hydroxyl ions and fluoride ions.

Map analysis of several massive fragments of fish debris (grain sizes range from 0.05 mm to 0.2 mm) reveals the presence of dentine, no enamel and an almost homogenous high REE content throughout the grain, contrary to the zoning seen in whole teeth. There is some variation in the REE concentrations in individual massive fragments (for example, the concentration of La is in the range 500–3,000 p.p.m.).

Zoning of REEs should occur in fish teeth because the enamel inhibits adsorption of REEs, whereas the apatite in dentine easily accepts REEs into the lattice. Biogenic apatite has many more lattice defects and impurities than does the apatite in igneous rock samples. Replacement and diffusion of REE ions in biogenic hydroxyapatite lattices can occur easily. Compared with dentine, enamel is extremely hard, highly crystalline and lacking in lattice defects and impurities^{21–23}. The dense structure of enamel is much less permeable to diagenetic exchange of REEs¹⁰.

Figure 3 shows the results of the analyses for La, Sm and Yb along line A–B of the grain shown in Fig. 2. REE concentrations increase from the tip (A) to the root of the grain (B).

We consider a simple one-dimensional model²⁴ of the diffusion of REEs through the dentine. Assuming that the source of the REEs is a thin film on the grain boundary of the root and that the diffusion coefficient D ($\text{mm}^2 \text{Myr}^{-1}$) of dentine is constant in all directions (dentine is polycrystalline) the equation of distribution of the REE content c (p.p.m. mm^{-1}) is $c = A/(2\sqrt{\pi Dt}) \exp(-x^2/4Dt)$ where x (mm) is the distance from the root (rim) of the tooth, t (Myr) is the time over which diffusion has occurred (~ 50 kyr) and A (p.p.m.) is the total REE content in the tooth which we calculate to be 150 p.p.m. mm by integrating the concentration curve of La along the transect.

We did calculations for two possible cases in which: (1) all of the La that eventually diffuses into the tooth is emplaced at the grain boundary at $t = 0$, that is, REE enrichment stops shortly after burial in the sediment; (2) the REE enrichment rate is constant from the time of deposition to the present.

Figure 4a, b shows the variations of concentration curves for various values of D in cases (1) and (2). Figure 4c compares

the La concentration curve with the curves from both cases that best approximate it—the curve of $D = 26$ ($\text{mm}^2 \text{Myr}^{-1}$) in case (1) and the curve of $D = 68$ ($\text{mm}^2 \text{Myr}^{-1}$) in case (2). The La concentration curve of the fish tooth is a different shape than those of the curves calculated from the diffusion equation. This may be because of the simplicity of a one-dimensional model and/or the elongated structure of fish teeth.

The sources of REEs in fish debris are considered to be: (1) REEs in the sea water through which the samples fall on their way to the sediment floor; (2) REEs in the overlying seawater column at the water/sediment interface; (3) REEs in pore sea water in the bioturbated and well ventilated uppermost layer; (4) REEs in diagenetic fluid in the surrounding sediment; (5) diagenetic REE transfer from detrital silicate to fish debris. As our samples were collected from the uppermost layer in oxic pelagic sediment, only the first three of these need to be considered. Sedimentation of fecal pellets from the surface to the sediment occurs on a timescale of a few days per 100 metres (ref. 25). Using the assumed value of D (26 – $68 \text{ mm}^2 \text{Myr}^{-1}$) and estimated sedimentation rate ($\sim 7 \text{ mm kyr}^{-1}$), it is difficult to explain the uptake of REEs into apatite during the falling of debris to the sediment floor and at the water/sediment interface. The significant variation of the La content (500–3,000 p.p.m. by EPMA) and La/Yb ratio (3.1–6.7 by INAA) among individual fish-debris grains may reflect the local variation of the amount of pore water that exchanges REEs with fish debris in pelagic sediment or variations in REE content of the pore water.

It is often suggested that the good correlation between REE content and P content in pelagic sediments is due to biogenic apatite in deep-sea sediment^{12,26,27}. Our estimation of D , 26 – $68 \text{ mm}^2 \text{Myr}^{-1}$, is consistent with that view^{4,12,27,28}.

The fish-tooth sample used in the ion-probe study¹⁰ was 7.5-mm long and its dentine had a homogeneously high REE content (for example, the La content was in the range 1,248–1,675 p.p.m.). Assuming that the diffusion coefficient D of dentine in that sample was of the order of $50 \text{ mm}^2 \text{Myr}^{-1}$, the REE content of those samples is too high to have originated only in REEs of the sea water in the overlying water column and in the uppermost layer of the sediment. The samples were deposited in the sandy inshore sediment over 43 Myr ago (ref. 4). Contrary to the case of samples from oxic pelagic sediments, it is presumed both that a great volume of water exchanged REEs with the fish debris in the sandy sediment and that the high REE content of pore water in near-shore anoxic sediments^{29–30} made possible the subsequent acquisition of REEs from the pore water but not from silicate particulates during the diagenetic process⁴. An elapsed time of 32 Myr was long enough and REE content in

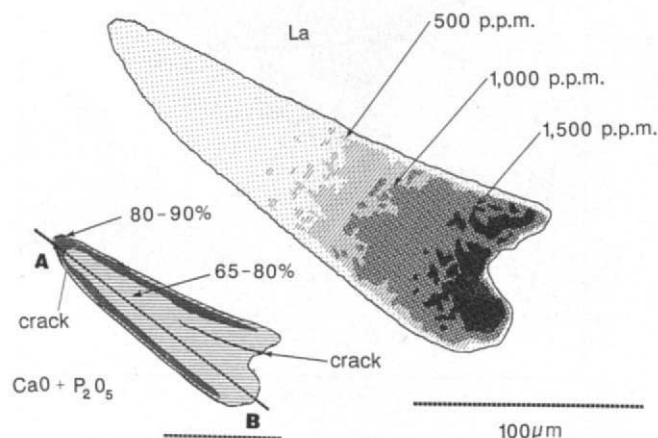


FIG. 2 Compositional zoning of La and $\text{CaO} + \text{P}_2\text{O}_5$ measured in thin sections parallel to the lineation of fish-teeth grains.

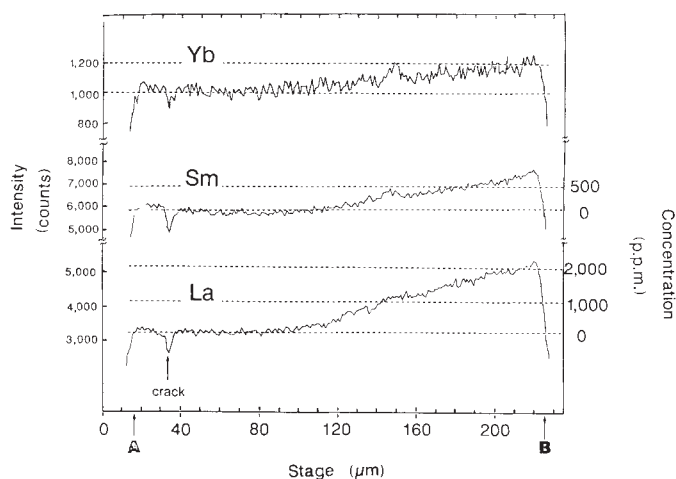


FIG. 3 Line profiles of La, Sm and Yb in fish teeth. The traverses were made along the line A–B in Fig. 2.

the pore water high enough to permit homogenization of the REE content in the dentine of that sample.

In contrast to the suggestion¹⁰ that REE uptake by the sedimented biogenic debris takes place essentially on the sediment and that late diagenesis is of marginal importance, we believe that REE uptake during late diagenetic processes (case 2) can be more important than REE uptake from the overlying water column (case 1). The REEs in our fish-debris samples were acquired from fluids in and near the surface sediment, not from the overlying column of sea water. Case (2) of the model is more suitable than case (1) for our estimated value of D , $\sim 70 \text{ mm}^2 \text{ Myr}^{-1}$. More data needs to be considered, however, in the case of near-shore sediments.

We conclude that REE data of fish debris from near-shore sediments and sandy sedimentary rocks should be used with caution as a palaeoceanographic indicator. □

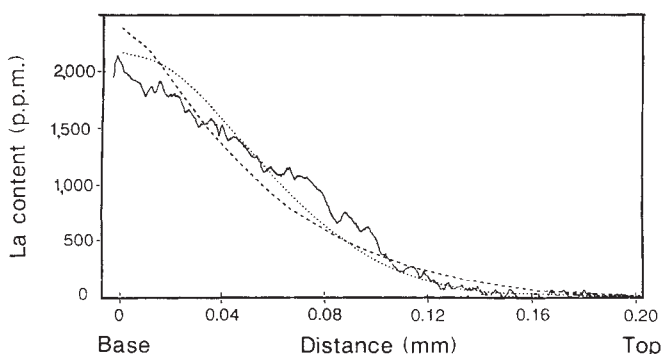
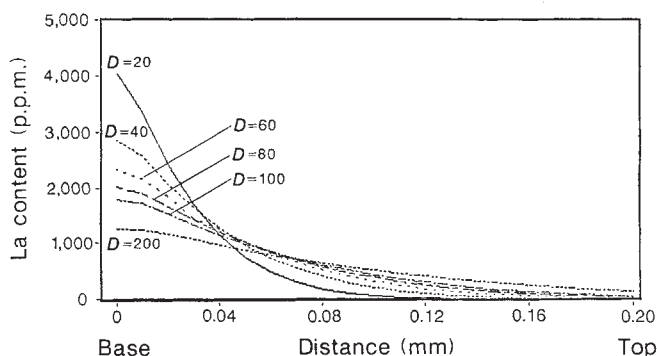
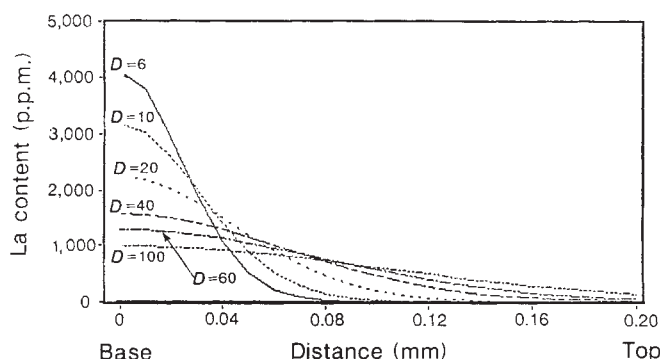


FIG. 4 Chemical zoning of La in the dentine of fish teeth calculated using a one-dimensional diffusion model: a, Calculated diffusion profiles when all the La is present at the grain boundary before diffusion starts; b, Calculated diffusion profiles when La is acquired at a constant rate; c, Comparison of the calculated curves (dotted line, $D = 26$, case (1); dashed line, $D = 68$, case (2)), and La concentration profile from line analysis (solid line).

Received 16 February; accepted 24 April 1990.

1. Wright, J., Schrader, H. & Holser, W. T. *Geochim. cosmochim. Acta* **51**, 631–644 (1987).
2. Shaw, H. F. & Wasserburg, G. J. *Geochim. cosmochim. Acta* **49**, 503–518 (1985).
3. Staudigel, H., Doyle, P. & Zindler, A. *Earth planet. Sci. Lett.* **76**, 45–56 (1985/86).
4. Grandjean, H., Cappetta, H., Michard, A. & Albarede, F. *Earth planet. Sci. Lett.* **84**, 181–196 (1987).
5. Grandjean, P., Cappetta, H. & Albarede, F. *Geophys. Res. Lett.* **15**, 389–392 (1988).
6. Tlig, S., Sassi, A., Belayouni, H. & Michel, D. *Chem. Geol.* **62**, 209–221 (1987).
7. Oudin, E. & Cocherie, A. *Geochim. cosmochim. Acta* **52**, 177–184 (1988).
8. Bernat, M. *Cathiers ORSTOM Series Geol.* **7**, 65–83 (1975).
9. Arrhenius, G., Bramlett, M. N. & Picciotto, E. *Nature* **180**, 85–86 (1957).
10. Grandjean, P. & Albarede, F. *Geochim. cosmochim. Acta* **53**, 3179–3183 (1989).
11. Kobayashi, K., Tonouchi, S., Furuta, T. & Watanabe, M. *Bull. Ocean Res. Inst., Univ. Tokyo* **13**, 55–64 (1980).
12. Toyoda, K., Nakamura, Y. & Masuda, A. *Geochim. cosmochim. Acta* **54**, 1093–1103 (1990).
13. Jaffrezic, H., Joron, J. N., Treuil, M. & Wood, D. A. *J. radioanal. Chem.* **55**, 417–425 (1980).
14. Piper, D. Z. *Chem. Geol.* **14**, 285–304 (1974).
15. Høgdahl, O. T., Melson, S. & Bowen, V. *Adv. Chem. Ser.* **73**, 308–325.
16. de Baar, H.-J. W., Bacon, M. P., Brewer, P. G. & Bruland, K. W. *Geochim. cosmochim. Acta* **49**, 1943–1959 (1985).
17. Roeder, P. L., MacArthur, D., Ma, X.-P., Palmer, G. R. & Mariano, A. N. *Am. Mineral.* **72**, 801–811 (1987).
18. Ozawa, K. *Nature* **338**, 141–143 (1989).
19. Berkovitz, B. K. B. et al. *Teeth* (Springer, Berlin, 1989).
20. Hodge, H. C. in *Oral History and Embryology* (ed. Silcher, H.) 53 (Mosby, St Louis, 1962).
21. Young, R. A. & Elliott, J. C. *Archs oral Biol.* **11**, 699–707 (1966).
22. Parthe, E. & Rieger, W. *J. dent. Res.* **47**, 829–835 (1968).
23. McCormell, D. J. *dent. Res.* **31**, 53–63 (1952).
24. Shewmon, P. G. *Diffusion in Solids* (McGraw-Hill, New York, 1963).
25. Fowler, S. et al. *Nature* **329**, 56–58 (1987).
26. Elderfield, H., Hawkesworth, C. J., Greaves, M. J. & Calvert, S. E. *Geochim. cosmochim. Acta* **45**, 513–528 (1981).
27. Piper, D. Z. *Geochim. cosmochim. Acta* **52**, 2127–2145 (1988).
28. Elderfield, H. & Pagett, R. *Sci. tot. Envir.* **49**, 175–197 (1986).
29. Elderfield, H. & Sholkovitz, E. R. *Earth planet. Sci. Lett.* **82**, 280–288 (1987).
30. Sholkovitz, E. R., Piepgras, D. J. & Jacobsen, S. B. *Geochim. cosmochim. Acta* **53**, 2847–2856 (1989).

ACKNOWLEDGEMENTS. We thank Professor Kushiro and the Radioisotope Center of the University of Tokyo for arranging the instruments, and H. Yoshida for help with the EPMA measurements.

Thiophenic biomarkers for palaeoenvironmental assessment and molecular stratigraphy

Jaap S. Sinninghe Damsté, Math E. L. Kohnen & Jan W. de Leeuw

Organic Geochemistry Unit, Faculty of Chemical Technology and Materials Science, Delft University of Technology, de Vries van Heystplantsoen 2, NL-2628 RZ Delft, The Netherlands

RECONSTRUCTION of ancient depositional environments is an important objective in the Earth sciences. The complex assemblages of organic molecules in sediments include compounds that can be related to known biochemicals produced by specific biota from well defined habitats^{1–3}, and these 'chemical fossils' are applied frequently to reconstruct palaeoenvironments, especially in studies of organic and petroleum geochemistry and in chemical oceanography. Most of the compounds used in this way are hydrocarbons^{2–4}, although oxygenated (for example, alcohols, acids and ketones) and nitrogen-containing (for example, porphyrins) compounds have also been applied successfully^{1,2}. Here we report on the use of organic sulphur compounds as geochemical tools for palaeoenvironmental and stratigraphic assessment. Depth profiles of specific sulphur compounds related to certain organisms show significant variations reflecting changes both in sources of organic matter and in physical conditions (such as salinity) of the depositional environment. In a number of cases these variations are not otherwise revealed by changes in lithology, geochemical bulk data and/or absolute and relative amounts of hydrocarbon biological markers. Moreover, several organic sulphur compounds reveal the presence of unprecedented biochemicals in these palaeoenvironments.

Significant developments in the understanding of the abiogenic formation of organically bound sulphur in the geosphere have occurred recently^{5,6}. The identification of organic sulphur compounds (OSCs) whose carbon skeletons bear an

unambiguous link with those of natural products indicate that inorganic sulphur species (such as H_2S and polysulphides) react with functionalized lipids at the earliest stages of diagenesis⁵⁻¹³. A prerequisite for the formation of OSCs is the presence of free H_2S (or polysulphides), which requires specific sedimentary conditions (sulphate reduction, low availability of iron)¹¹. The early incorporation of inorganic sulphur species into labile organic substrates generates OSCs that preserve the carbon skeletons and information concerning the original sites of functionalities of their lipid precursors¹⁰. This latter information is lost in the corresponding hydrocarbon products. Because the lipid signatures of sediments strongly depend on the composition of biota in the sedimentary environment and on several environmental factors, retrieval of this information is useful in the assessment of palaeoenvironments. The OSCs produced are thought to be much more stable than their biological precursors under geological conditions so this information may be carried through even to ancient sediments¹⁰. It was therefore suggested that OSCs are potential biomarkers¹¹. Here we test whether OSCs can reveal palaeoenvironmental changes by a detailed investigation of 13 samples from a borehole in the Jurf ed Darawish Oil Shale Basin from Jordan.

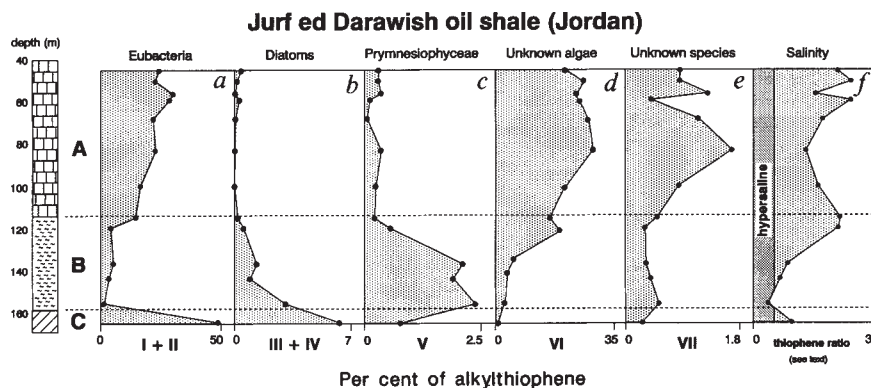
These immature organic-rich sediments were deposited on a continental shelf of the Tethys Sea during the Upper Cretaceous^{14,15}. Three lithological facies can be recognized (Fig. 1): a bituminous limestone (45–115 m), a bituminous calcareous marl (115–160 m) and a phosphorite (160–170 m). Geochemical bulk data and absolute amounts and distribution patterns of hydrocarbon biological markers did not reveal any major variations in the composition of the organic matter related to palaeoenvironmental changes in the fossil record other than those revealed by the lithology itself¹⁶. For example, a higher

detrital influx during the deposition of facies B was recognized by the presence of a marked terrigenous component in the sediment as revealed by the relatively higher amounts of odd-carbon-numbered *n*-alkanes in the C_{25} – C_{33} range (biological markers for vascular plants) and the lower $\delta^{13}C$ values ($\delta^{13}C = ((^{13}C/^{12}C)_{\text{sample}} / (^{13}C/^{12}C)_{\text{standard}}) - 1$) of the organic matter. The higher clay content of the sediments in this lithological unit is, however, also reminiscent of the higher detrital influx.

In contrast with other geochemical signatures the distributions of the alkylthiophenes in solvent extracts from samples of this deposit show marked changes which allow a substantial refinement of the stratigraphy¹⁶. Figure 1 shows the depth profiles of a number of selected OSCs. The C_{35} thiophene hopanoids I (ref. 7) and II (ref. 11) (Fig. 2) are formed by sulphur incorporation into bacteriohopanetetrol, a constituent of the cell membranes of many prokaryotes^{17,18}, and can therefore be used as bacterial markers. The relatively high amounts of thiophenes I and II in facies C (Fig. 1a) is in full agreement with the presumption that phosphatization results from a high turn-over rate of the organic matter caused by bacterial reworking^{19,20}. The much lower relative amounts of these bacterial markers in facies B indicate less extensive bacterial reworking which is consistent with the inferred euxinic palaeodepositional conditions of this lithological unit¹⁶. Facies A has been deposited under less extreme reducing conditions¹⁶ and, therefore, shows an intermediate relative hopanoid thiophene content.

The C_{25} highly branched isoprenoid thiophenes III and IV (Fig. 2) are thought to result from incorporation of sulphur into widely occurring C_{25} alkenes biosynthesized by diatoms^{11,12}, although it is as yet unknown whether the occurrence of the C_{25} alkenes is restricted exclusively to this class of algae^{21,22}. The depth profile of these thiophenes (Fig. 1b) reveals a sharp

FIG. 1 Lithological column and depth profiles of a, C_{35} hopanoid thiophenes I+II; b, C_{25} highly branched isoprenoid thiophenes III+IV; c, C_{37} 2,5-dialkylthiophenes V; d, C_{44} – C_{48} 3,4-dialkylthiophenes VI; e, C_{26} irregular isoprenoid thiophene VII; f, thiophenes VIIa–b/VIIIc–g. Samples analysed are composite 1-m-long samples from a drill-hole core. Lithology was assessed by whole-rock analyses (for major elements) using emission spectroscopy and from literature data¹⁴. Carbonate content was determined separately by treatment with HCl. Alkylthiophenes were separated from extracts (obtained by sonication using mixtures of CH_3OH and CH_2Cl_2) by column chromatography followed by argentation thin-layer chromatography^{10,16}. Alkylthiophene fractions were analysed by gas chromatography/mass spectrometry (GC-MS). GC conditions: Hewlett-Packard instrument with on-column injection, fitted with a 25 m $\times$ 0.32 mm CP Sil-5 (film thickness 0.1 μm) fused-silica capillary column, temperature programmed from 50 to 120 $^{\circ}C$ at 10 $^{\circ}C$ min^{-1} and then to 320 $^{\circ}C$ (30-min hold time) at 4 $^{\circ}C$ min^{-1} . MS conditions: VG-70S instrument, ionization voltage 70 eV, mass range m/z 40–800, cycle time



1.8 s. Alkylthiophenes were quantified by integration of mass chromatograms of their molecular ions and that of the internal standard, 5-(1,1-d₂-hexadecyl)-2,3-dimethylthiophene¹⁶. Analytical reproducibility is derived from three duplicate analyses and is estimated to be >90%.

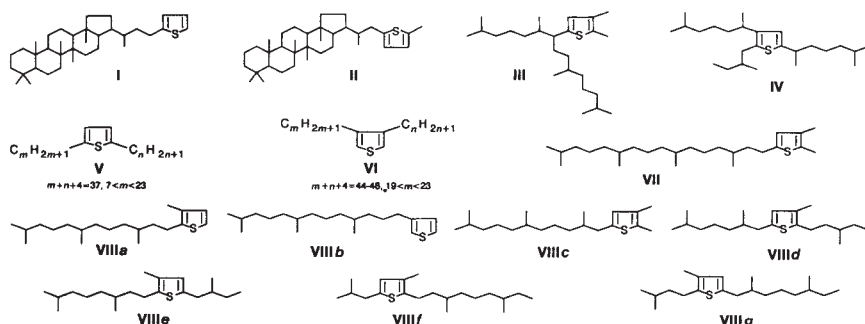


FIG. 2 Structures of compounds.

increase in the lower part of facies B and in facies C thus clearly inferring a relatively larger contribution of diatoms to the sedimentary organic matter in this zone. This is consistent with the fact that phosphatic sediments are often deposited in upwelling environments¹⁹ and that the phytoplankton in such environments tends to be dominated by diatoms³.

A depth profile (Fig. 1c) of the C₃₇ 2,5-dialkylthiophenes V (Fig. 2) shows that Prymnesiophyte algae (which include coccolithophorids) were predominant during deposition of the lower section of facies B. Thiophenes V are formed by reaction of inorganic sulphur species with the C₃₇ di- and triunsaturated methyl- and ethylketones (and/or their corresponding alkenes)^{10,11} biosynthesized by this group of algae and used in the assessment of palaeotemperatures²³. This depth profile is the only way of obtaining information on the occurrence of Prymnesiophyceae because the ketones (or the corresponding alkenes) were not present as such. Similarly, a depth profile (Fig. 1d) of the C₄₄–C₄₈ 3,4-dialkylthiophenes VI²⁴ (Fig. 2) reveals the abundance of other, probably phytoplanktonic, species during deposition of facies A and the upper part of facies B. Compounds with these carbon skeletons have not been reported previously, indicating that their precursors are too labile to be preserved in the sedimentary record. It is only through their sulphur derivatives that their original presence is revealed. A depth profile (Fig. 1e) of the C₂₆ irregular isoprenoid thiophene VII²⁵ (Fig. 2) shows two maxima in the upper section of facies A. This is a further example of the recognition of hitherto unrecognized lipids by analysis of OSCs as C₂₆ irregular isoprenoids are not known in nature. Many other changes in the alkylthiophene assemblages were noted and seem to contain further stratigraphic information¹⁶. For a number of these OSCs, however, it is not yet known how they relate to specific biological contributions. This is, in the main, because of our scant knowledge of the lipid composition of phytoplankton³, although the possibility remains that the organism capable of biosynthesizing suitably functionalized lipid precursors for these OSCs have become extinct.

C₂₀ isoprenoid thiophene distributions may be used in the assessment of palaeosalinity^{11,26}. A depth profile (Fig. 1f) of the ratio of thiophenes VIIIa^{8,27} and VIIIb⁸ over VIIIc-g^{28,29} (Fig. 2) shows small variations in palaeosalinity. Values smaller than 0.5 have been proposed to reflect hypersaline (salinity >4%) palaeoenvironments²⁶. Thus, this depth profile reveals that the palaeosalinity in this sedimentary sequence has only been higher than that of normal sea water during deposition of the lowest part of facies B. In a separate study of a related deposit it was also concluded that the lowermost beds were deposited in a hypersaline environment³⁰. This example shows that alkylthiophenes can also be used in the assessment of physical aspects of the depositional palaeoenvironment. Furthermore, the parallel changes in the depth profiles of the thiophene ratio and thiophenes VI indicate that the unknown algae biosynthesizing the precursors of VI have been stressed by enhanced salinities. Also, the maximum in the depth profile of thiophenes V (abundance of Prymnesiophyceae) occurs at the highest salinity. This is consistent with the palaeoenvironmental changes observed in the Black Sea where the coccolith *E. Huxleyi* appears as marine incursions replace lacustrine depositional conditions³¹. □

7. Valisilalao, J., Perakis, N., Chappe, B. & Albrecht, P. *Tetrahedron Lett.* **25**, 1183–1186 (1984).
8. Brassell, S. C., Lewis, C. A., de Leeuw, J. W., de Lange, F. & Sinninghe Damsté, J. S. *Nature* **320**, 160–162 (1986).
9. Vairavamurthy, A. & Mopper, K. *Nature* **329**, 623–625 (1987).
10. Sinninghe Damsté, J. S., Rijpstra, W. I. C., Kock-van Dalen, A. C., de Leeuw, J. W. & Schenck, P. A. *Geochim. cosmochim. Acta* **53**, 1343–1355 (1989).
11. Sinninghe Damsté, J. S., Rijpstra, W. I. C., de Leeuw, J. W. & Schenck, P. A. *Geochim. cosmochim. Acta* **53**, 1323–1341 (1989).
12. Sinninghe Damsté, J. S., van Koert, E. R., Kock-van Dalen, A. C., de Leeuw, J. W. & Schenck, P. A. *Org. Geochem.* **14**, 555–567 (1989).
13. Kohnen, M. E. L., Sinninghe Damsté, J. S., ten Haven, H. L. & de Leeuw, J. W. *Nature* **341**, 640–641 (1989).
14. Hufnagel, H. *Geol. J., Rh A* **75**, 295–311 (1984).
15. Bein, A. & Amit, O. *Sedimentology* **29**, 81–90 (1982).
16. Kohnen, M. E. L., Sinninghe Damsté, J. S., Rijpstra, W. I. C. & de Leeuw, J. W. in *Geochemistry of Sulfur in Fossil Fuels* (eds Orr, W. C. & White, C. M.) ACS Symp. Ser. Vol. 429 (in the press).
17. Ourisson, G., Albrecht, P. & Rohmer, M. *Pure appl. Chem.* **51**, 709–729 (1979).
18. Ourisson, G., Albrecht, P. & Rohmer, M. *Trends biochem. Sci.* **7**, 236–239 (1982).
19. Bendor, Y. K. (ed.) *Marine Phosphorites—Geochemistry, Occurrence, Genesis, Shell Exploration en Productie Maatschappij Spec. Publ.* **29** (1980).
20. Isaacs, C. M., Piciotto, K. A. & Garrison, R. E. *Devl Sedimentol.* **36**, 247–282 (1983).
21. Rowland, S. J., Yon, D. A. & Maxwell, J. R. *Org. Geochem.* **8**, 207–213 (1985).
22. Nichols, P. D., Volkman, J. K., Palmisano, A. C., Smith, G. A. & White, D. C. *J. Phycol.* **24**, 90–96 (1988).
23. Brassell, S. C., Eglinton, G., Marlowe, I. T., Pflaum, U. & Sarnthein, M. *Nature* **320**, 129–133 (1986).
24. Kohnen, M. E. L., Peakman, T. M., Sinninghe Damsté, J. S. & de Leeuw, J. W. in *Advances in Organic Geochemistry 1989* (eds Durand, B. & Behar, F.) (Pergamon, Oxford, in the press).
25. Peakman, T. M., Sinninghe Damsté, J. S. & de Leeuw, J. W. *JCS Chem. Commun.* 1105–1106 (1989).
26. de Leeuw, J. W. & Sinninghe Damsté, J. S. in *Geochemistry of Sulfur in Fossil Fuels* (eds Orr, W. I. & White, C. M.) ACS Symp. Ser. Vol. 429 (in the press).
27. Rulkötter, J., Landrag, M. & Disko, U. *J. high res. Chrom. Commun.* **11**, 633–638 (1988).
28. Sinninghe Damsté, J. S., Kock-van Dalen, A. C., de Leeuw, J. W. & Schenck, P. A. *Tetrahedron Lett.* **28**, 957–960 (1987).
29. Sinninghe Damsté, J. S. & de Leeuw, J. W. *Int. J. environ. analyt. Chem.* **28**, 1–19 (1987).
30. Spiro, B. & Aizenshtat, Z. *Nature* **269**, 235–237 (1977).
31. Bukry, D. in *The Black Sea—Geology, Chemistry and Biology* (eds Degens, E. T. & Ross, D. A.) 353–363 (Am. Ass. Petrol. Geol., Tulsa, 1974).

ACKNOWLEDGEMENTS. We thank H. Wehner for samples, W. I. C. Rijpstra for analytical assistance and R. Kreulen for measurements of carbon isotopes. This work was partly supported by the Netherlands Foundation for Earth Science Research with financial aid from the Netherlands Organisation for Scientific Research.

Replacement of pyrite framboids by magnetite in limestone and implications for palaeomagnetism

D. Suk, D. R. Peacor & R. Van der Voo

Department of Geological Sciences, University of Michigan, Ann Arbor, Michigan 48109, USA

LIMESTONES are an important source of palaeomagnetic data, with magnetite the dominant carrier of the magnetization. Because the magnetite is present in low concentrations, however, it has been directly observed only rarely and in trace amounts in acid-treated separates. Such residues are composed primarily of spheres, the ultimate origin of which has been enigmatic⁷. Yet interpretation of the palaeomagnetic data depends on a knowledge of the origin of the magnetite, which has been inferred to carry either a chemical or a viscous remanent magnetization. Here we present electron microscope data obtained from a sequence of samples of Onondaga Limestone from an east–west traverse across northern New York, which unambiguously show that magnetite spheres are derived by alteration and replacement of framboidal pyrite. Remagnetization is thus caused by chemical, rather than viscous, processes. This chemical remanent magnetization is compatible with a fluid-mediated event occurring on a regional scale, induced by tectonic stress of Alleghenian (late Palaeozoic) age.

It is widely recognized that many of the early to middle Palaeozoic shallow-water and platform carbonates in eastern north America were remagnetized during the Kiaman reversed superchron of the late Palaeozoic^{1–8} and examples of late Mesozoic remagnetizations are now beginning to be recognized in the western United States^{9,10}. The ancient remagnetizations are presumably related to the tectonism in space and time^{6,9–12}, and tectonically driven fluid migrations from active plate margins may have an important role in the remagnetization processes¹³.

Received 30 November 1989; accepted 20 April 1990.

1. Johns, R. B. (ed.) *Biological Markers in the Sedimentary Record* (Elsevier, Amsterdam, 1986).
2. Brassell, S. C. & Eglinton, G. in *Organic Marine Geochemistry* (ed. Sohn, M. L.) 10–32 (Am. chem. Soc., Washington, DC, 1986).
3. Volkman, J. K. in *Lacustrine Petroleum Source Rocks* (eds Fleet, A. J., Kelts, K. & Talbot, M. R.) 103–112 (Blackwell, Oxford, 1988).
4. Mackenzie, A. S. *Adv. Petrol. Geochem.* **1**, 115–214 (1984).
5. Sinninghe Damsté, J. S., Rijpstra, W. I. C., de Leeuw, J. W. & Schenck, P. A. in *Advances in Organic Geochemistry 1987* (eds Novelli, L. & Matavelli, L.) 593–606 (Pergamon, Oxford, 1988).
6. Sinninghe Damsté, J. S. & de Leeuw, J. W. in *Advances in Organic Geochemistry 1989* (eds Durand, B. & Behar, F.) (Pergamon, Oxford, in the press).

Two fundamentally different explanations for remagnetization have been proposed. The first explanation involves a chemical remanent magnetization (CRM) acquired when new magnetic minerals (primarily magnetite) formed long after the formation of the rocks. The other explanation invokes a change in magnetic direction in pre-existing magnetic minerals, known as viscous remanent magnetization (VRM). Although far from definitive, recent work on the same or similar platform carbonates in the eastern United States^{4,5,8,14} has generally produced more evidence in favour of CRM than of VRM. The magnetic extracts used in some studies were shown to consist in large part of spheres of magnetite. They were never found *in situ*, however, so the ultimate origin of such spheres was undetermined. Scanning and transmission electron microscope (SEM and TEM) observations of thin sections of the lower Ordovician carbonate Knox Group of east Tennessee revealed some mineralogical and textural relationships of iron oxides with their *in situ* surroundings¹⁵, but the source of the spheres of magnetite remained unclear.

In an attempt to determine directly the processes of remagnetization, middle Devonian Onondaga limestone has been sampled along a profile across New York from Kingston via Albany to Buffalo. Magnetic susceptibility increases towards the east, with magnetization throughout the sequence being of Alleghenian age, and hypothesized to be tectonically induced. Rock samples were prepared for SEM observation in the form of thin sections attached by sticky wax. Areas containing iron oxides, as first identified by SEM, were removed from sections and ion milled. A Hitachi S-570 SEM and a Philips CM-12 STEM (scanning tunnelling electron microscope), both equipped with Kevex Quantum energy dispersive analysis (EDA) systems, were used for all electron microscope observations with operating conditions as described by Jiang *et al.*¹⁶.

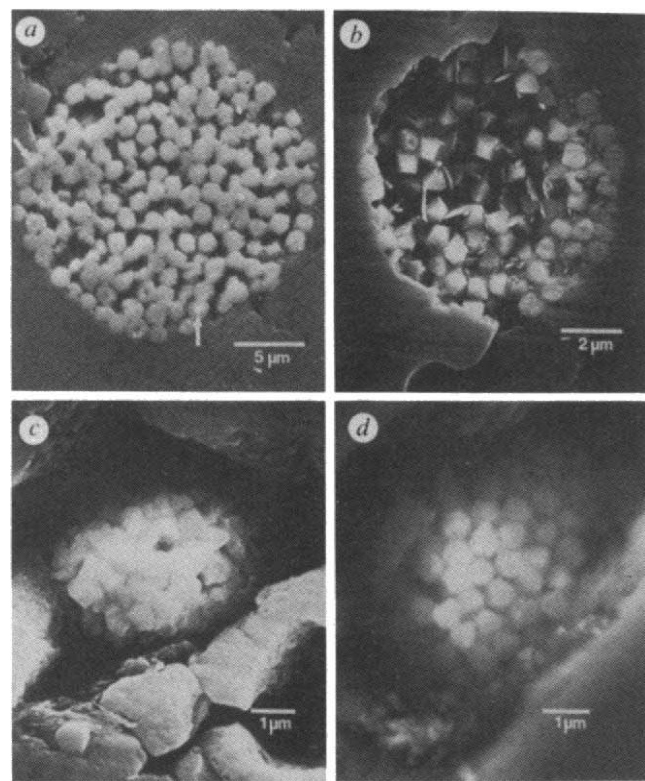


FIG. 1 Secondary-electron images of magnetite that replaced framboidal pyrite in Onondaga limestone. *a*, Polished cross-section of pseudoframboid in calcite matrix. Bright cores of some crystals (arrow) are remnant pyrite. *b*, Cross-section of pseudoframboidal magnetite showing individual octahedral or cubo-octahedral grains. *c*, Pseudoframboid in crack in calcite. *d*, Pseudoframboid showing close packing of crystals.

Figure 1 shows a secondary-electron image (SEI) of a polished thin section containing magnetite that replaced framboidal pyrite, as typical of most SEM observations of this study. Identification of this specific grain assemblage as magnetite is tentative as it is directly based on EDA spectra showing only Fe and O (see Fig. 2*d*). In all cases where such pseudoframboids were ion milled and examined by TEM (Fig. 2*a* and 2*b*), however, selected-area electron diffraction (SAED) patterns showed that magnetite is the dominant phase, sometimes accompanied by haematite. Areas consisting of magnetite are polycrystalline in nature, based on the spotty and diffuse nature of SAED patterns which approximate the appearance of powder photographs (Fig. 2*c*). In addition, magnetic extracts were recovered from the insoluble residue obtained by dissolving limestones in buffered acetic acid of pH 5 (refs 3, 4). SEM images of grains in magnetic extracts of acid-treated samples seemed to be identical to those obtained from *in situ* grains, although there is a possibility of alteration of magnetic extracts during acid treatment. The grains from magnetic extracts gave X-ray powder diffraction patterns principally of magnetite, with no other iron compounds. Therefore, where EDA spectra of samples observed by SEM show only Fe and O, a specific grain is labelled as magnetite by association, although there may be haematite in some cases.

As shown in Fig. 1*b*, pseudoframboids consist of arrays of individual crystals less than 1 µm in diameter, the form of which is dominantly octahedral or cubo-octahedral; a polished cross-section of such crystals shown in Fig. 1*a*. Figure 1*c* shows a pseudoframboid in a small crack, separated by pore space from carbonate matrix. The crystal packing arrangement, where one crystal is surrounded on average by 12 neighbours that commonly share crystal faces, is illustrated in Fig. 1*d*. Individual crystals also occur, randomly arrayed in the carbonate matrix (Fig. 3). As shown in Figs 1*a* and 3*b*, the cores of some crystals consist of unaltered pyrite (brighter phase) with magnetite rims whereas cores of other crystals are hollow, due either to plucking of the pyrite during specimen preparation or dissolution of pyrite (Fig. 3*b*).

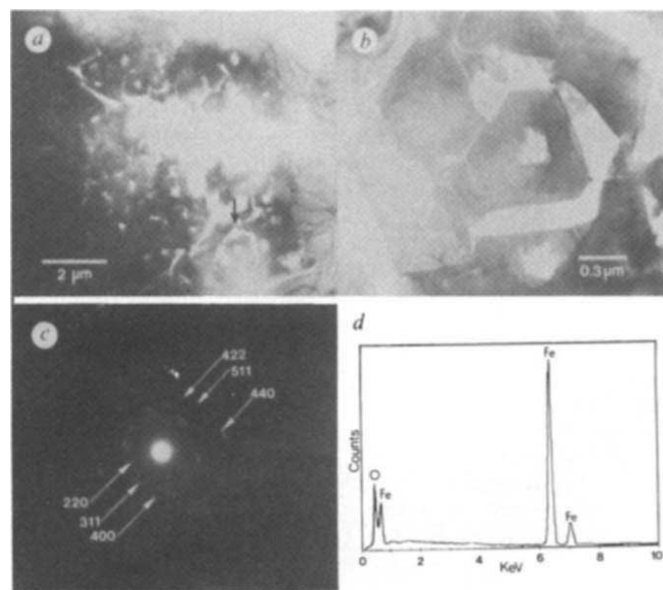


FIG. 2 Bright-field images of magnetite, selected-area electron diffraction (SAED) pattern and energy dispersive spectrum obtained by AEM. *a*, Part of pseudoframboidal magnetite ion milled for TEM observations. *b*, Enlarged magnetite grains from the lower right side (arrow) in *a*. *c*, Typical SAED patterns of magnetite were obtained from these individual grains. *d*, Energy dispersive spectrum of grain shown in *a*, identical to energy dispersive spectra obtained by SEM.

Unaltered pyrite framboids are more common than those consisting of magnetite. Only pyrite has been observed in samples from western New York, where the magnetic susceptibility of limestones is low. In contrast, magnetic extracts of samples from near Albany, for which SEM shows that magnetite is common, are dominated by spheres of magnetite (Fig. 4a). Such spheres have morphologies identical to those observed in polished thin sections and give rise to X-ray diffraction patterns showing only reflections due to magnetite (Fig. 4b). They are therefore inferred to be equivalent to the pseudoframboidal magnetite observed in thin section. Such spheres seem to be identical to those observed only in separates in many other studies^{3,4,9}, the origins of which were obscure.

Canfield and Berner¹⁷ have shown that pyrite framboids can form by pyritization of magnetite in unconsolidated sediments. Our observations demonstrate conclusively that the reverse reaction can occur much later and that the magnetic phase that is the principal source of remanent magnetism has replaced early pyrite. The remagnetization is therefore a chemical remanent magnetization (CRM) acquired when pseudoframboidal magnetite formed by reactions involving pyrite in the presence of fluids. Fluid flow in the remagnetization processes is thought to be driven by tectonic activity¹³ on a continental scale. Alternatively, sediment compaction¹⁸ and gravity due to topographic difference¹⁹ in the intracratonic basins can be important driving forces for basinal fluid flows as well. Such basinal fluids consist either of connate fluid expelled by sediment compaction, or meteoric water introduced by gravity owing to uplift of sur-

rounding regions. Tectonically driven fluids, consisting of connate fluids, may then be mixed with a later meteoric water component¹³. Because the remagnetization of limestones throughout the eastern United States has been inferred to be largely due to spherical magnetite as observed in separates^{3,4}, and because our pseudoframboids have appearances nearly identical to those in separates of other studies, we conclude that replacement of framboids and individual crystals of pyrite by magnetite is the principal process responsible for remagnetization in such rocks. Elliott and Aronson²⁰ have determined that the smectite of bentonites in the southern Appalachian Basin has undergone alteration to illite over a narrow time interval (between 272 and 303 Myr). Similarly, Hearn *et al.*²¹ have shown that authigenic K-feldspar occurring in limestone along the flanks of the entire Ouachita–Appalachian fold belt formed by the reaction of connate brines during the Alleghenian orogeny. These relations are consistent with the Alleghenian age of chemical remagnetization residing in authigenic magnetite. All of these mineralogical transitions are inferred to be mediated by fluids, and are consistent with recrystallization of all minerals in a single event, hypothesized by Oliver¹³ to be caused by tectonically induced crustal fluid flow. □

Received 12 January; accepted 24 April 1990.

1. Kent, D. V. *J. geophys. Res.* **84**, 3576–3588 (1979).
2. Scotese, C.R., Van der Voo, R. & McCabe, C. *Phys. Earth planet. Inter.* **30**, 385–395 (1982).
3. McCabe, C., Van der Voo, R., Peacor, D. R., Scotese, C. R. & Freeman, R. *Geology* **11**, 221–223 (1983).
4. Bachtadse, V., Van der Voo, R., Haynes, F. M. & Kesler, S. E. *J. geophys. Res.* **92**, 14165–14176 (1987).
5. Jackson, M., McCabe, C., Ballard, M. M. & Van der Voo, R. *Geology* **16**, 592–595 (1988).
6. Miller, J. D. & Kent, D. V. *Geology* **16**, 588–591 (1988).
7. McCabe, C. & Elmore, D. R. *Rev. Geophys.* **27**, 471–494 (1989).
8. Jackson, M. *J. geophys. Res.* **95**, 2753–2761 (1990).
9. McWhinnie, S. T., van der Pluijm, B. A. & Van der Voo, R. *J. geophys. Res.* **95**, 4551–4559 (1990).
10. Globerman, B. R. & Irving, E. *J. geophys. Res.* **93**, 11721–11733 (1988).
11. Hodych, H. J. *Geophys. Res. Lett.* **16**, 93–96 (1989).
12. Van der Voo, R. *Geol. Soc. Am. Mem.* **172**, 447–470 (1989).
13. Oliver, J. *Geology* **14**, 99–102 (1986).
14. McCabe, C., Jackson, M. & Saffer, B. *J. geophys. Res.* **94**, 10429–10443 (1989).
15. Suk, D., Van der Voo, R. & Peacor, D. R. *J. geophys. Res.* (in the press).
16. Jiang, W. T., Essene, E. J. & Peacor, D. R. *Clays Clay Miner.* (in the press).
17. Canfield, D. E. & Berner, R. A. *Geochim. cosmochim. Acta* **51**, 645–659 (1987).
18. Noble, E. A. *Econ. Geol.* **58**, 1145–1156 (1963).
19. Garven, G. & Freeze, R. A. *Am. J. Sci.* **284**, 1085–1124 (1984).
20. Elliott, W. C. & Aronson, J. L. *Geology* **15**, 735–739 (1987).
21. Hearn, P. P. Jr, Sutter, J. F. & Belkin, H. E. *28th Int. Geol. Congr. Abstr. Vol.*, 2-46–2-47 (1989).

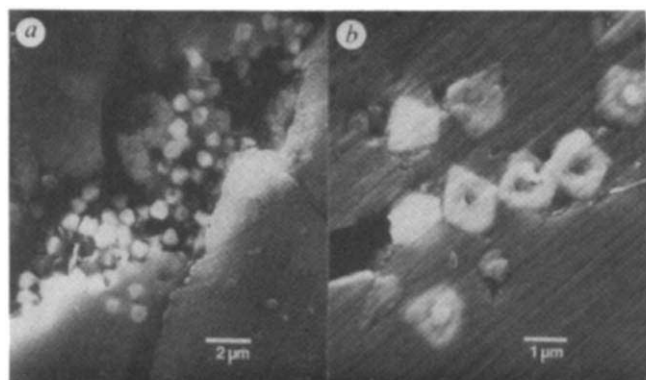


FIG. 3 Secondary-electron images of magnetite that replaced individual pyrite crystals. *a*, Crystals dispersed in calcite matrix. *b*, Crystals with remnant pyrite cores (bright contrast) and hollow cores (dark contrast).

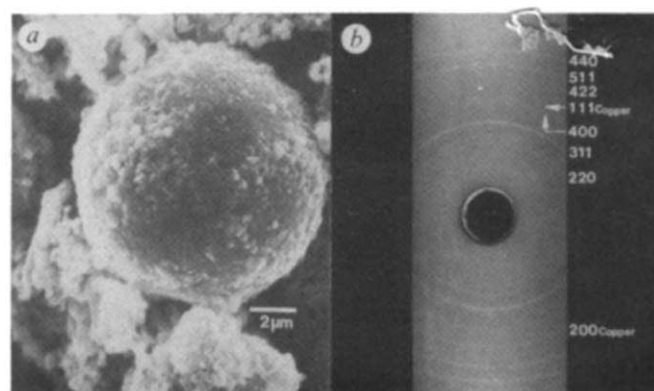


FIG. 4 Magnetite sphere from separates obtained by dissolution of calcite and X-ray diffraction pattern. *a*, Sphere showing typical irregular surface. *b*, X-ray diffraction pattern obtained using Gandolfi Camera showing 220, 311, 400, 422, 511 and 440 reflections of magnetite, and 111 and 200 reflections of copper from the collimator.

Mineral nutrition and seasonal movements of African migratory ungulates

S. J. McNaughton

Biological Research Laboratories, Syracuse University, Syracuse, New York 13244–1220, USA

Two distinct foraging behaviours differentiate Africa's grazing ungulates^{1–3}: over the wet–dry season cycle resident herds occupy seasonally stable and spatially restricted home ranges and migratory herds move over vast geographical regions, using different areas in different seasons. Migratory herds dominate animal biomass wherever they occur⁴. Some migrants congregate around permanent water sources in the dry season and disperse to drier habitats in the wet season⁵; others travel along rainfall gradients, concentrating in areas of low annual rainfall in the wet season and moving to areas of higher rainfall as the dry season advances⁴. The Serengeti National Park in Tanzania is typical of the latter type of ecosystem⁶; migratory grazers there select drier localities in the wet season, despite the fact that forage production rates and drinking water supplies are high everywhere. These migrations could be explained by the animals' avoidance of muddy, sticky soils⁷, general forage-quality properties⁸, by calcium requirements

of lactating females⁹ and by avoidance of woodland habitats with greater predation risk¹⁰. Resident grazers are concentrated in localities supporting grasses with mineral contents more capable of meeting dietary requirements than forages in nearby, little-used grasslands¹¹. I now present evidence that seasonal movements of migratory grazers in the Serengeti ecosystem are also related to grass mineral content. Particularly important, based on forage concentrations and animal requirements¹², are Ca, Cu, N, Na, Zn and also, for lactating females and growing young, Mg, P and Ca/P balance.

The youngest fully expanded leaf blades of the dominant grass species and paired upper 10-cm soils were sampled at 115 locations throughout Serengeti National Park during the wet season and treated as described previously¹¹. Collection locations were classified as wet, transitional, or dry-season areas based on well-documented movements of migratory herds⁶. Wet-season areas are on the arid south eastern Serengeti Plains, dry-season areas are in the subhumid northern part of the Park, and transitional areas are in between. In addition to elements known to be important in animal nutrition, some of importance to plants but not to animals (for example, boron) and some not known to have functional roles in either except at toxic levels (for example, cadmium) were also measured. Two elemental ratios were considered: Ca/P because it influences the availability of Ca and P to ungulates; and Cu/Mo as it affects Cu availability¹².

Two discriminant functions separated grasslands used in different seasons with high resolution (Fig. 1). The first function ($\chi^2 = 223$, degrees of freedom = 40, $P < 0.00001$) separated wet-season grasslands from all others, and the second ($\chi^2 = 56$, d.f. = 19, $P = 0.0002$) separated transitional and dry-season vegetation. Thus, seasonal usage by the migratory ungulates is reflected in forage mineral content during the wet season. Dry- and wet-season ranges are completely distinct nutritionally.

Elements loading heavily and positively on the first axis were, in order of the standardized discriminant function coefficients (SDFC), Fe, N, Mg and Ca (Table 1). V and Na are high negative values on this axis; K and Na load heavily and positively on the second axis; and Ca/P and Cu score high negative SDFC. Wet-season areas support forages of consistently higher mineral content (Table 2). Exceptions to this are Cd, K, Mo, Ni and Se, which do not vary; this is important as none of these is likely to be nutritionally significant at the levels measured. Forages from the dry-season range were high in Mn and Zn and those from transitional areas were high in Cu, Mg, Na and P.

Analysis of the distribution of resident herds provides no indication that soil mineral levels contribute to forage properties¹¹, but the grasslands considered here occur along a fertility gradient from ancient, infertile granitic soils in the dry-season range to recent, fertile volcanic soils in the wet-season range^{8,13}. Of the forage mineral properties apparently important to

seasonal usage patterns, all but Mg are significantly associated with soil levels (Table 2). Therefore, underlying soil fertility patterns, particularly with respect to N, Na and P, contribute to forage mineral properties that are related to seasonal usage by migratory herds.

Animal nutrition is a complex process: both nutrient requirement and availability vary with animal and forage properties¹². Nevertheless, ungulate requirements are broadly similar¹⁴ and nutritional deficiencies leading to veterinary symptoms have been extensively documented in wild African ungulates³. Using beef cattle standards¹², Serengeti grasses are deficient to marginal in Cu and Zn, dry-season forages are marginal in N and badly deficient in Na, non-wet-season forages are deficient in Ca for lactating and growing animals, and dry-season forages are deficient in Mg and P for lactating and growing animals. Observed Cu/Mo ratios are unlikely to be important. The optimum Ca/P ratio is believed to be between 1 and 2, and the dry-season forages exceed that ratio, so that P deficiency might be exacerbated. Apart from Na, which is supplemented everywhere, P-deficiency is the most prevalent livestock deficiency throughout the world¹².

Seasonal ranges are flexible and defined by average annual usage. In the wet season, migrants move to transitional areas during periods of rainfall failure and even to dry-season areas during extended periods of rainfall failure elsewhere⁶. Neither do all animals in a herd move together. Lactating females and calves of the principal migratory species, wildebeest (*Conno-*

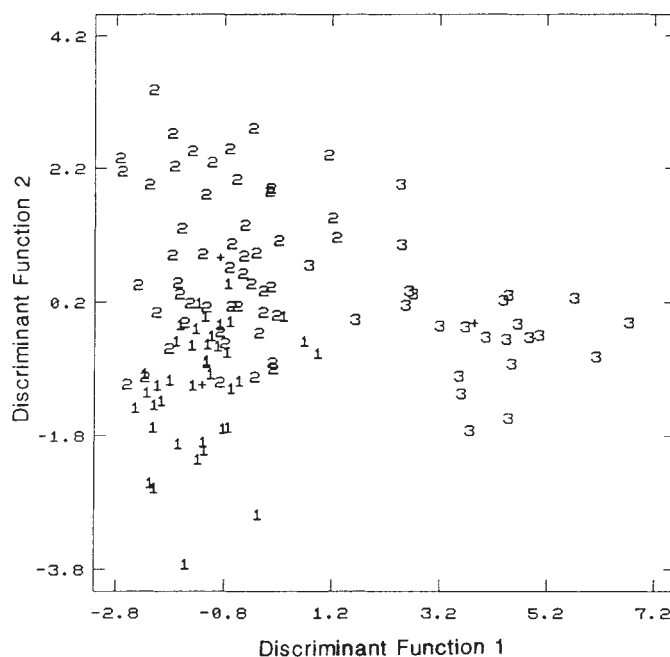


FIG. 1 Discriminant analysis of forages based on mean stand values ($N = 115$) of mineral content from different seasonal ranges of migratory ungulates in the Serengeti National Park, Tanzania. Key to ranges: 1, dry season; 2, transitional; 3, wet season. Group centroids: dry season stands $DF1 = -1.21$, $DF2 = -1.03$; transitional stands $DF1 = -0.87$, $DF2 = 0.87$; wet season stands $DF1 = 3.87$, $DF2 = -0.12$. Of 40 dry season stands, 36 were classified accurately on the basis of forage mineral content, the other 4 being classified as transitional. Of 51 transitional stands, 40 were classified as originally categorized, the remaining 11 being classified as dry-season vegetation. Of 24 wet season stands, all but 1 were accurately classified, that stand being classified as transitional. Samples of the youngest fully expanded leaf blades collected from 3–6 individuals of the dominant grass species at each location were processed and analysed as described previously¹¹. The level of N was determined by gas chromatography on a Carlo Erba 1500 N-analyser and all other elements were determined on a Leeman Labs PlasmaSpec 2.5 Inductively Coupled Argon Plasma Spectrometer in simultaneous mode.

TABLE 1 Standardized discriminant function coefficients

Element or ratio	DF1	DF2	Element or ratio	DF1	DF2
Al	0.28	-0.35	Mo	-0.18	-0.06
B	-0.02	0.10	N	0.69	-0.18
Ca	0.46	0.19	Na	-0.54	0.71
Cd	-0.04	-0.23	Ni	-0.37	-0.14
Co	-0.02	0.21	P	-0.08	0.14
Cu	0.06	-0.40	Se	0.05	0.19
Fe	0.77	0.36	V	-0.59	-0.22
K	-0.35	0.75	Zn	-0.25	-0.29
Mg	0.61	0.26	Ca/P	-0.27	-0.44
Mn	-0.27	-0.08	Cu/Mo	0.12	-0.23

Standardized discriminant function coefficients for forage elements and elemental ratios in different seasonal ranges of migratory grazing ungulates in the Serengeti National Park, Tanzania.

chaetes taurinus), move to the park's transitional areas when rains fail on the Serengeti Plains, but bulls and non-lactating cows often travel straight north into the dry season range. This divergent movement is probably related to growth and lactation demands, and forage levels of Cu, Mg, N, Na, P, and Ca/P.

One distinctive property of grasses in the Serengeti ecosystem is the existence of Na-accumulators ($\text{Na} > 1,000 \mu\text{g g}^{-1}$) that are particularly evident in transitional and wet season ranges. Resident herds are concentrated in high-Na areas¹¹ and grazer abundance in the Serengeti may be influenced by the presence of Na-accumulating grasses.

Minerals that are at elevated levels in grasses of wet-season and transitional ranges are required at substantially higher concentrations by females at late stages of pregnancy and in lactation and by growing young¹². Calving of wildebeest is synchronous, with the vast majority of calves born within a two-week period in mid-wet season¹⁵. Similar parturition peaks occur in migratory zebras (*Equus burchelli*), elands (*Taurotragus oryx*), and Thomson's gazelles (*Gazella thomsoni*)¹⁵. These birth peaks result in the greatest lactation demand when there is a high probability of sufficient rainfall to support the migratory herds on the SE Serengeti Plains¹³. Migrant reproductive patterns may have evolved under the constraint of nutritional stress on lactating females and calves imposed by births at other times.

The following conclusions are of importance to both ecosystem science and conservation policy. First, forage minerals are important to the seasonal movements of migratory grazing mammals, as well as influencing the spatial distribution of resident grazers¹¹. The migratory herds that dominate animal biomass in the Serengeti ecosystem move along a forage mineral gradient, counter to the rainfall gradient. Animals in more arid ecosystems, which disperse in the wet season and concentrate around permanent water sources in the dry season⁵, may also be responding to the spatial distribution of forage minerals. Secondly, the requirements of young, growing animals and of females that are lactating or in the late stages of pregnancy are particularly influential to the movements. This indicates that the animals have evolved parturition periods governed by the nutritional requirements of reproductive females and young animals,

seasonal rainfall patterns, and the spatial distribution of forage nutritional sufficiency.

Thirdly, those areas of Africa set aside as national parks and game reserves because of their abundant ungulates may all contain nutritionally critical habitats supporting these herds. Concentrations of forage minerals seem to be reliable indicators of critical habitats on both local and regional spatial scales. In addition, Na-accumulating grasses may constitute an important, hitherto overlooked, constituent of ecosystems supporting abundant large grazing mammals. Should animal reintroduction become an important conservation policy¹⁶, surveys of forage mineral properties might be useful guides to the design of ecosystem reconstruction projects. □

Received 13 March; accepted 17 April 1990.

1. Delany, M. J. & Happold, D. C. D. *Ecology of African Mammals* (Longman, London, 1979).
2. Eltringham, S. K. *The Ecology and Conservation of Large African Mammals* (Macmillan, London, 1980).
3. McNaughton, S. J. & Georgiadis, N. J. *A. Rev. Ecol. Syst.* **17**, 39–65 (1986).
4. Fryxell, J. M. *et al. Am. Nat.* **131**, 781–798 (1988).
5. Western, D. E. *Afr. Wildlife J.* **13**, 265–286 (1975).
6. Maddock, L. in *Serengeti. Dynamics of an Ecosystem*. (eds Sinclair, A. R. E. & Norton-Griffiths, M.) 104–129 (Univ. Chicago, Chicago, 1979).
7. Ancerson, G. D. & Talbot, L. M. *J. Ecol.* **53**, 33–56 (1965).
8. Bell, R. H. V. in *Ecology of Tropical Savannas* (eds Huntley, B. J. & Walker, B. H.) 193–216 (Springer, New York, 1982).
9. Kruegen, D. E. *Afr. Wildlife J.* **13**, 297–304 (1975).
10. Darling, F. F. *Wildl. Monogr.* **5** (1960).
11. McNaughton, S. J. *Nature* **334**, 343–345 (1988).
12. McDowell, L. R. (ed.) *Nutrition of Grazing Ruminants in Warm Climates* (Academic, New York, 1985).
13. McNaughton, S. J. *Ecol. Monogr.* **55**, 259–294 (1985).
14. Taylor, St. C. S. & Murray, J. I. in *The Nutrition of Herbivores* (eds Hacker, J. B. & Ternouth, J. H.) 487–533. (Academic, Sydney, 1987).
15. Schaller, G. B. *The Serengeti Lion* (Univ. Chicago, Chicago, 1972).
16. Stanley Price, M. R. in *Conservation for the Twenty-first Century* (eds Western, D. & Pearl, M.) 210–8 (Oxford, New York, 1989).

ACKNOWLEDGEMENTS. I thank R. S. McNaughton, N. J. Georgiadis, F. F. Banyikwa, R. W. Ruess, and B. Maas for help with the collection of samples. Laboratory analyses were performed by M. M. McNaughton, M. Oesterheld and K. J. Williams. I also thank the trustees and director of Tanzania National Parks and the National Scientific Research Council of Tanzania for permission to live and do research in the Serengeti National Park and officers of the Serengeti Wildlife Research Institute for research facilities and housing. This research was supported by the NSF.

TABLE 2 Mean elemental concentrations in grass leaf blades from different seasonal ranges of migratory ungulates in the Serengeti

Element or ratio	Concentrations ($\mu\text{g g}^{-1}$)			F	P<
	Dry	Transitional	Wet		
Al	166a	207a	807b	104	0.0001
B	4.95a	5.80b	7.79b	6.92	0.002
Ca	4.026a	3.811a	6.159b	24.5	0.0001
Cd	0.04a	0.03a	0.05a	2.94	NS
Co	0.205a	0.298a	0.40b	14.3	0.0001
Cu	5.54a	6.14ab	7.08b	4.64	0.02
Fe	107a	140b	417c	92	0.0001
K	17,209a	20,007a	19,887a	1.88	NS
Mg	1,667a	1,772ab	1,945b	3.82	0.03
Mn	91.0b	58.8a	62.7ab	6.17	0.003
Mo	2.46a	2.46a	2.77a	1.13	NS
N	15,600a	18,600b	25,200c	23.8	0.0001
Na	151a	2,943b	933b	19.2	0.0001
Ni	0.83a	0.87a	0.79a	0.23	NS
P	2,701a	4,039b	4,440b	19.3	0.0001
Se	1.00a	1.16a	1.07a	0.5	NS
V	1.45a	1.51a	2.01b	13.0	0.0001
Zn	19.2ab	17.1a	22.2b	8.3	0.0001
Ca/P	2.16b	1.14a	1.45ab	15.6	0.0001
Cu/Mo	4.29a	3.19a	3.49a	0.8	NS

The F test and Scheffe range tests were done on natural logarithm-transformed values with degrees of freedom of 2 and 112 in all cases. All concentrations are in $\mu\text{g g}^{-1}$ on a dry-weight basis. Values not followed by the same letter are significantly different at least at $P=0.05$. Correlations of nutritionally significant grass minerals with soil mineral levels, using Spearman's rank correlation coefficient were (d.f.=113 for all): Ca, $r=0.337$, $P<0.04$; Cu, $r=0.278$, $P=0.003$; Mg, $r=0.106$, $P=\text{not significant}$; N, $r=0.445$, $P<0.0001$; Na, $r=0.540$, $P<0.0001$; P, $r=0.525$, $P<0.0001$; Zn, $r=0.193$, $P=0.039$; and Ca/P, $r=0.247$, $P<0.01$. NS, not significant.

Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding

Paul A. Roche & Peter Cresswell

Department of Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina 27710, USA

CLASS II major histocompatibility complex (MHC) molecules are heterodimeric cell surface glycoproteins which bind and present immunogenic peptides to T lymphocytes. Such peptides are normally derived from protein antigens internalized and proteolytically degraded by the antigen-presenting cell¹. Class I MHC molecules also bind immunogenic peptides, but these are derived from proteins synthesized within the target cell². Whereas class I molecules seem to bind peptides in the endoplasmic reticulum^{3–5}, class II molecules are thought to bind peptides late in transport. Intracellular class II molecules associate in the endoplasmic reticulum with a third glycoprotein, the invariant (I) chain, which is proteolytically removed before cell surface expression of the $\alpha\beta$ class II heterodimer^{6,7}. It has been suggested that the I chain prevents peptides from associating with class II molecules early in transport⁸. Preventing such binding until the class II molecules enter an endosomal compartment could maintain the functional dichotomy between class I and class II MHC molecules. We have examined the ability of I chain-associated HLA-DR5 molecules

to bind a well characterized influenza haemagglutinin-derived peptide (HAP). The results show that whereas mature HLA-DR $\alpha\beta$ dimers effectively bind this peptide, the I chain-associated form does not.

Purified mature HLA-DR5 $\alpha\beta$ dimers were incubated with ^{125}I -labelled HAP (*HAP) in detergent solution and free *HAP was separated from HLA-DR-bound *HAP by high-performance size-exclusion chromatography (HPSEC). DR5 molecules bind this peptide in a specific, saturable manner with high affinity ($K_D \sim 20$ nM; ref. 9). Figure 1a shows that when purified $\alpha\beta$ dimers were incubated with *HAP under pseudo-first order reaction conditions, a peak of radioactivity appeared which coeluted with the $\alpha\beta$ dimers of relative molecular mass $\sim 70,000$ ($M_r = 70\text{K}$). The inset to Fig. 1a shows a Coomassie blue-stained two-dimensional gel of the $\alpha\beta$ preparation showing the sample contained only HLA-DR α - and β -chains. Immunoprecipitation of this material with an anti-class II antiserum and an anti-I chain antiserum also confirmed that the class II-bound *HAP was not associated with the I chain (see below). Using the known specific activity of the *HAP preparation, a binding stoichiometry of $1.2 \text{ pmol } ^*\text{HAP } \mu\text{g}^{-1} \alpha\beta$ was calculated (Table 1). Assuming that each $\alpha\beta$ dimer is capable of binding only one *HAP molecule, about 8% of the HLA-DR molecules in the preparation had bound *HAP after 15 h of incubation, in close agreement with our previous values for *HAP binding to isolated HLA-DR molecules⁹.

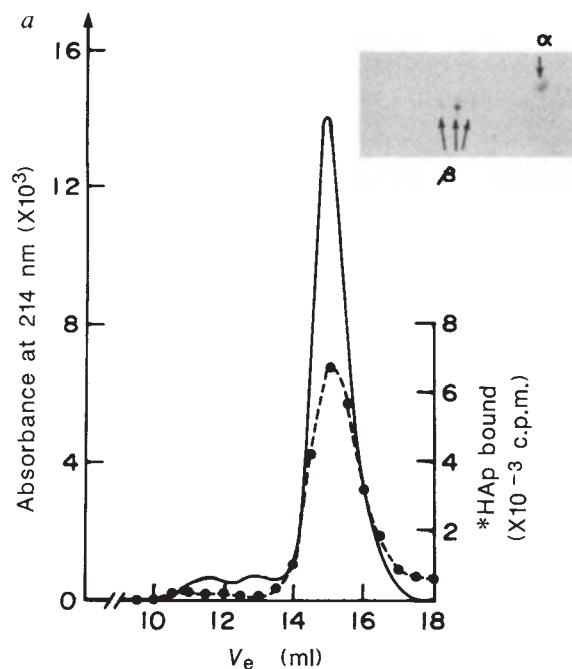
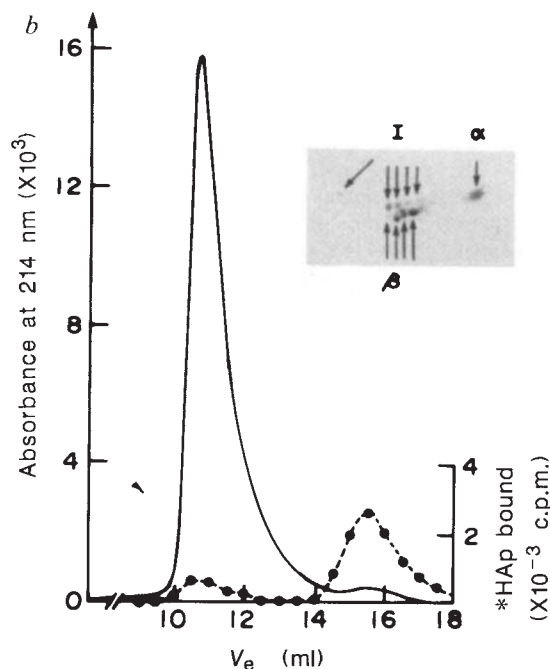


FIG. 1 Binding of *HAP to $\alpha\beta$ and $\alpha\beta\text{I}$ complexes. *a*, Purified mature DR5 $\alpha\beta$ dimers ($1.5 \mu\text{g}$) or *b*, immature DR5 $\alpha\beta\text{I}$ complexes ($1.5 \mu\text{g}$), were incubated with 200 nM *HAP in HPSEC buffer (50 mM sodium phosphate, 1% n -octyl- β -D-glucopyranoside, $\text{pH } 7.0$) containing 1 mM iodoacetamide. After incubation at room temperature (15 h), the samples were analysed by HPSEC using a TSK G3000 SW size exclusion chromatography column ($7.5 \text{ mm} \times 600 \text{ mm}$) and the radioactivity in each 0.5 ml fraction detected in a gamma-counter. The protein absorbance at 214 nm (solid line) and radioactivity present in each fraction (dashed line) were plotted as a function of elution volume in ml (V_e). Free *HAP eluted in the included volume of the column as a distinct peak at 26 ml and is not shown. The high M_r of the $\alpha\beta\text{I}$ complex ($>200\text{K}$) has been noted previously^{10,14}, and could be a consequence of the association of the α - and β - chains with an anomalously large I chain, rather than the association of the $\alpha\beta\text{I}$ complex with a proteoglycan molecule²⁰. The inset of each panel shows a Coomassie blue-stained 2-dimensional gel of the protein ($\sim 10 \mu\text{g}$) in each preparation. The position of the DR α -, β -, and I chain spots are indicated by arrows. The complexity

Figure 1b shows the binding of *HAP to purified immature HLA-DR5 ($\alpha\beta\text{I}$) complexes. The inset shows a Coomassie blue-stained two-dimensional gel of the preparation which confirms our previous finding that the DR α -, β -, and I chains are present in approximately equimolar amounts in immature DR preparations¹⁰. When $\alpha\beta\text{I}$ complexes were incubated with *HAP for 15 h and analysed by HPSEC, there was only a very small amount of *HAP associated with the material in the high M_r $\alpha\beta\text{I}$ peak. Although the I chain-associated form of HLA-DR was essentially incapable of binding *HAP, there was a significant amount of *HAP associated with the small amount of $\alpha\beta$ dimer present in the preparation (Fig. 1b).

To calculate the relative *HAP binding ability of the I chain-associated form of HLA-DR, the amount of *HAP bound per μg protein in both the $\alpha\beta\text{I}$ and $\alpha\beta$ peaks was determined (Table 1). Unlike the lower M_r $\alpha\beta$ dimers in this preparation, the high M_r $\alpha\beta\text{I}$ complex bound *HAP extremely poorly. Only 0.05 pmol *HAP was bound per μg $\alpha\beta\text{I}$ complex, which corresponds to $<0.3\%$ of the molecules in the preparation having bound *HAP. In contrast, 13.5 pmol *HAP was bound per μg $\alpha\beta$ dimer in this same preparation, which corresponds to *HAP binding to 94% of the molecules in this population. As all traces of mature $\alpha\beta$ dimers were removed from the initial immature DR5 ($\alpha\beta\text{I}$) preparation, and because dramatically different *HAP saturation levels were obtained between the 'mature' $\alpha\beta$ dimers and the $\alpha\beta$ dimers seen in Fig. 1b (8% against 94% occupancy of



of the pattern is a result of both incomplete glycosylation and the use of alternative start sites in the translation of I chain mRNA (reviewed in ref. 14). METHODS. The procedures used for the growth of the HLA-DR5 homozygous B-lymphoblastoid cell line Swei, isolation of mature $\alpha\beta$ dimers, radiolabelling of HAP, HPSEC, and 2-dimensional gel electrophoresis have been described^{6,9}. To purify immature $\alpha\beta\text{I}$ complexes, lysates from monensin-treated Swei cells were first passed over an immobilized L243 (anti-DR $\alpha\beta$) immunoaffinity column to remove mature $\alpha\beta$ dimers^{15,16} and $\alpha\beta\text{I}$ complexes then were isolated by immunoaffinity chromatography using an immobilized-DA6.147 (anti-DR α) affinity column¹⁷. The complexes were eluted from this column at $\text{pH } 11$, neutralized with 1 M glycine, and subjected to additional purification by HPSEC⁹. Both preparations were contaminant free as determined by SDS-PAGE and Coomassie blue staining. Swei cells were treated with $10 \mu\text{M}$ monensin prior to collecting to allow the intracellular accumulation of large amounts of $\alpha\beta\text{I}$ complexes^{9,18}. Control experiments produced essentially identical results with $\alpha\beta$ and $\alpha\beta\text{I}$ complexes isolated from cells grown in the presence or absence of monensin.

the HLA-DR molecules by *HAp), we believe that the latter were spontaneously released from the $\alpha\beta$ I complex during the incubation with *HAp and were not contaminants of mature $\alpha\beta$ dimers. Regardless of their origin, however, these calculations show that, on a weight basis, there is a 200-fold increase in the ability of *HAp to bind to the $\alpha\beta$ dimers as compared with the $\alpha\beta$ I complexes in the immature DR5 ($\alpha\beta$ I) preparation.

To rule out the possibility that the lack of *HAp binding was a consequence of the irreversible denaturation of the $\alpha\beta$ I complex during the isolation procedure, we attempted to deliberately dissociate functional $\alpha\beta$ dimers from the high M_r $\alpha\beta$ I complex. HPSEC-purified $\alpha\beta$ I complexes were incubated with *HAp in the presence or absence of 0.1% SDS for 20 h and subsequently analysed by HPSEC. Incubation of $\alpha\beta$ I complexes with *HAp in the absence of SDS (Fig. 2a) resulted in only minimal peptide binding, and once again the majority of the protein-bound *HAp eluted at the position of $\alpha\beta$ dimers. By contrast, incubation of the $\alpha\beta$ I complex with *HAp in the presence of SDS resulted in a dramatic change in the HPSEC elution profile and a marked increase in *HAp binding to a species which eluted at the position characteristic of the $\alpha\beta$ dimer (Fig. 2b). Control experiments demonstrated that 0.1% SDS had no effect on the ability of mature $\alpha\beta$ dimers to bind *HAp (data not shown). The profile of the chromatogram and the corresponding anti-DR α - and β -chain western blots confirmed that there was a dramatic increase in the amount of α - and β -chains present in the *HAp-associated fractions. Interestingly, whereas SDS treatment effectively dissociated $\alpha\beta$ dimers from the high M_r $\alpha\beta$ I complex, it did not affect the elution position of the I chain. The nature of the residual high M_r I chain complex is currently under investigation.

To prove that the *HAp binding species consisted of HLA-DR molecules, samples from the higher and lower M_r peaks were treated with either anti-class II-, anti-I chain-, or control-antisera and immunoprecipitated. Figure 3 shows that the small amount of *HAp present in the high M_r position in the control and SDS-treated preparations could be immunoprecipitated with both anti-class II and anti-I chain antisera, whereas *HAp which eluted at the lower M_r position could only be immunoprecipitated with the anti-class II antiserum. Also evident is the dramatic increase in *HAp binding to the $\alpha\beta$ dimer which is liberated by SDS treatment of the $\alpha\beta$ I complex. These results confirm that free $\alpha\beta$ dimer is the predominant *HAp binding species even when present in trace amounts in the $\alpha\beta$ I preparation and suggest that the $\alpha\beta$ dimer has a much greater affinity for *HAp than does the $\alpha\beta$ I complex.

Essentially all of the $\alpha\beta$ dimers spontaneously released from the $\alpha\beta$ I complex had bound *HAp after 15 h, whereas only 8% of the $\alpha\beta$ dimers in the mature DR5 preparation had bound *HAp under identical conditions. This is most readily explained

by the hypothesis that *HAp binding to spontaneously released $\alpha\beta$ dimers simply involves occupancy of an empty peptide binding groove on the $\alpha\beta$ dimer, whereas *HAp binding to mature $\alpha\beta$ dimers involves dissociation of endogenous peptides from the occupied peptide binding groove before *HAp binding to the $\alpha\beta$ dimer can occur. However, the $\alpha\beta$ dimer liberated from the $\alpha\beta$ I complex by SDS treatment ($\alpha\beta_{\text{SDS}}$) bound 5.2 pmol *HAp per μg protein, corresponding to only 36% saturation of

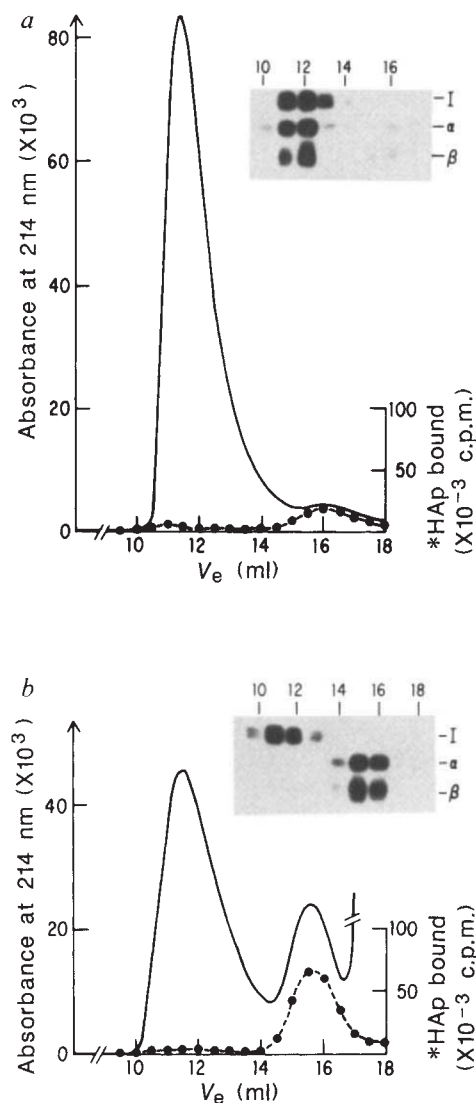


FIG. 2 Effect of SDS on *HAp binding to $\alpha\beta$ I complexes. HPSEC-purified $\alpha\beta$ I complexes ($10 \mu\text{g}$) were treated with 200 nM *HAp in the absence (a) or presence (b) of 0.1% SDS in HPSEC buffer containing 1 mM iodoacetamide. After incubation at room temperature (20 h), the samples were analysed by HPSEC and the radioactivity determined as in Fig. 1. Control experiments confirmed that the peak eluting at ~ 17 ml contained SDS molecules present in *n*-octyl- β -D-glucopyranoside micelles. The inset of each panel is a composite showing anti-HLA-DR α -, anti-class II β -, and anti-I chain western blots of alternate column fractions.

METHODS. Following gamma counting, alternate column fractions were precipitated with 7 volumes of ice cold ethanol, lyophilized, and reconstituted in SDS sample buffer. Following boiling for 3 min, equal volumes of each sample were loaded onto identical SDS-PAGE gels and protein electrophoretically transferred to nitrocellulose. One blot was probed with a 1:1 mixture of anti-HLA-DR α -chain (DA6.147)¹⁷ and anti-class II β -chain (XD5.A11)¹⁹ monoclonal antibodies and a ^{125}I -labelled rat monoclonal to mouse kappa-chain (OX-20)¹¹. The other blot was probed with a 1:50 dilution of a rabbit anti-I chain antiserum (Matilda)²⁰ and ^{125}I -labelled Protein A. The class II α -, β -, and I chains were visualized by fluorography using Kodak X-Omat film and intensifying screens.

TABLE 1 Binding of *HAp to various HLA-DR5 molecules

HLA-DR species	*HAp bound† (pmol μg^{-1})	HLA-DR occupied‡ (%)
Mature $\alpha\beta$	1.2 ± 0.6	8
Immature $\alpha\beta$ I	0.05 ± 0.02	0.3
Immature $\alpha\beta_{\text{spont}}^{\S}$	13.5 ± 5.0	94
Immature $\alpha\beta_{\text{SDS}}^{\parallel}$	5.2 ± 1.2	36

† The amount of protein present in the $\alpha\beta$ I and $\alpha\beta$ peaks was estimated by comparing the area of the peak to that of the BSA standard. The amount of *HAp bound was calculated using the known specific activity of the *HAp preparation ($21,400 \text{ c.p.m. pmol}^{-1}$). The average $\pm$ one standard deviation of three separate experiments is shown.

‡ The percentage of HLA-DR molecules occupied by *HAp was calculated assuming that each HLA-DR molecule ($M_r = 70\text{k}$) is capable of binding only one molecule *HAp and that each $\alpha\beta$ I complex contains one HLA-DR $\alpha\beta$ dimer.

$\S$ Spontaneously released from immature HLA-DR $\alpha\beta$ I complexes.

$\parallel$ Released by treatment of immature HLA-DR $\alpha\beta$ I with SDS.

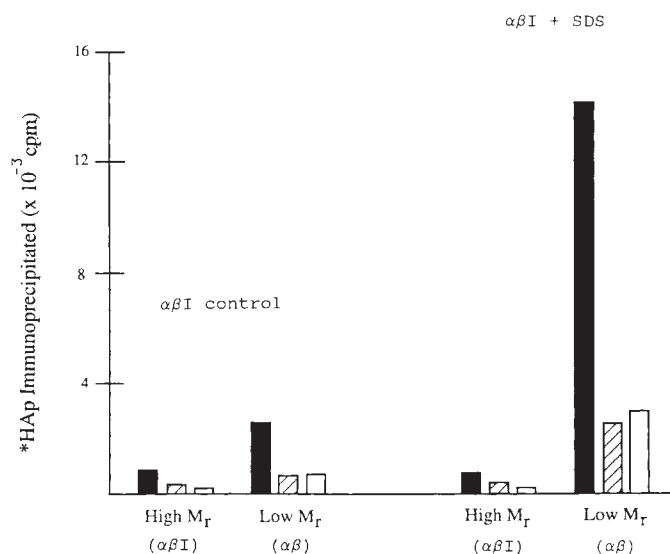


FIG. 3 Immunoprecipitation of *HAp binding proteins with anti-class II antisera. Purified $\alpha\beta$ I complexes were treated with *HAp in the absence or presence of SDS as described in Fig. 2. The material eluting at either the high M_r $\alpha\beta$ I position ($V_e=11$ ml) or the lower M_r $\alpha\beta$ position ($V_e=15$ ml) was pooled and equal volumes were incubated with an anti-class II antiserum (solid bar), an anti-I chain antiserum (hatched bar) or normal mouse serum (open bar). Antibody-bound *HAp was then immunoprecipitated with immobilized Protein A and radioactivity determined in a gamma-counter. The amount of *HAp immunoprecipitated by the anti-class II antiserum was approximately 40% of the total radioactivity present in each sample.

METHODS. Following HPSEC of the $\alpha\beta$ I/*HAp reaction mixture as described in Fig. 2, the material which eluted at 10.5 and 11.5 ml (the high M_r $\alpha\beta$ I position) and 14.5 and 15.5 ml (the lower M_r $\alpha\beta$ position) were pooled separately. Immunoprecipitation of the material in each pool with the anti-class II antiserum 247_{HSB} (ref. 18), the anti-I chain antiserum (Matilda), or normal rabbit serum was performed using Protein A-Sepharose (Sigma) in HPSEC buffer⁶.

the molecules in the population. The decreased amount of *HAp binding to $\alpha\beta_{SDS}$ as compared to spontaneously released $\alpha\beta$ dimers may be a result of partial denaturation of the $\alpha\beta_{SDS}$ molecules, altered association kinetics between *HAp and spontaneous or SDS-induced $\alpha\beta$ dimers, or the possibility that $\alpha\beta$ dissociation from $\alpha\beta$ I, and not *HAp binding, is the rate limiting step in the formation of an *HAp- $\alpha\beta_{SDS}$ complex.

There are two likely possibilities for the lack of peptide binding to I chain-associated $\alpha\beta$ dimers. One is that the I chain itself sterically hinders access of peptides to the binding cleft in the immature $\alpha\beta$ I complex. Alternatively, the $\alpha\beta$ dimers in the complex may be in such a conformation that binding of peptides to the cleft is not possible. For example, the peptide binding cleft of the immature $\alpha\beta$ I complex may not be properly formed, or access to the cleft may be sterically inhibited by regions of the DR α - or β -chains themselves. Regardless of the exact mechanism, our results suggest that association with the I chain prevents the binding of endogenous (self) peptides to class II molecules, such as those which would be encountered during transport of class II molecules to endosomal compartments. As class I molecules seem to bind immunogenic peptides very early³⁻⁵ and class II molecules are thought to bind immunogenic peptides much later in transport, the I chain may therefore serve to maintain the functional dichotomy between class I molecules and class II molecules.

We have shown that the isolated I chain-associated form of HLA-DR does not effectively bind an immunogenic peptide. As the I chain is proteolysed and dissociates from the class II $\alpha\beta$ dimer in an acidic, proteinase-containing compartment⁷, these results suggest that immunogenic peptides bind to class II

molecules in an endosomal compartment only after dissociation of the I chain from the $\alpha\beta$ I complex. In support of this hypothesis are results that demonstrated the intersection of the endocytic pathway with $\alpha\beta$ I complexes¹¹, the inhibitory effects of proteinase inhibitors on both I chain dissociation and antigen processing^{7,12}, and recent electron microscopic evidence colocalizing DR $\alpha\beta$ dimers, I chain, internalized immunoglobulin, and the proteinases cathepsin B and D in endosome-like vesicles in human B-lymphoblastoid cell lines¹³. □

Received 18 January; accepted 29 March 1990.

1. Unanue, E. R. A. *Rev. Immun.* **2**, 395-428 (1984).
2. Townsend, A. R. M. & Bodmer, H. A. *Rev. Immun.* **7**, 601-624 (1989).
3. Nuchtern, J. G., Bonifacio, J. S., Biddison, W. E. & Klausner, R. D. *Nature* **339**, 223-226 (1989).
4. Yewdell, J. W. & Bennink, J. R. *Science* **244**, 1072-1075 (1989).
5. Townsend, A. R. M. *et al. Nature* **340**, 443-448 (1989).
6. Machamer, C. E. & Cresswell, P. *J. Immun.* **129**, 2564-2569 (1982).
7. Blum, J. S. & Cresswell, P. *Proc. natn. Acad. Sci. U.S.A.* **85**, 3975-3979 (1988).
8. Elliot, W. L., Stille, C. J., Thomas, L. J. & Humphreys, R. E. *J. Immun.* **138**, 2949-2952 (1987).
9. Roche, P. A. & Cresswell, P. *J. Immun.* **144**, 1849-1856 (1990).
10. Kelner, D. N. & Cresswell, P. *J. Immun.* **137**, 2632-2639 (1986).
11. Cresswell, P. *Proc. natn. Acad. Sci. U.S.A.* **82**, 8188-8192 (1985).
12. Takahashi, H., Cease, K. B. & Berzofsky, J. A. *J. Immun.* **142**, 2221-2229 (1989).
13. Guagliardi, L. E. *et al. Nature* **343**, 133-139 (1990).
14. Cresswell, P., Blum, J. S., Kelner, D. N. & Marks, M. S. *CRC Crit. Rev. Immun.* **7**, 31-54 (1987).
15. Lampson, L. A. & Levy, R. *J. Immun.* **125**, 293-299 (1980).
16. Cresswell, P. & Blum, J. S. *Processing and Presentation of Antigens* (eds Pernis, B., Silverstein, S. C. & Vogel, H. J.) 43-51 (Academic, San Diego, 1988).
17. Guy, K., van Heyningen, V., Cohen, B. B., Deane, D. L. & Steel, C. M. *Eur. J. Immun.* **12**, 942-948 (1982).
18. Machamer, C. E. & Cresswell, P. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1287-1291 (1984).
19. Radka, S. F., Machamer, C. E. & Cresswell, P. *Hum. Immun.* **10**, 177-188 (1984).
20. Marks, M. S., Blum, J. S. & Cresswell, P. *J. Cell Biol.* (in the press).

ACKNOWLEDGEMENTS. We thank Alan Payne for assistance in preparative growth of cell lines and photography. This work was supported by the NIH.

Kinetics, stoichiometry and role of the Na-Ca exchange mechanism in isolated cardiac myocytes

Lynn M. Crespo, Christopher J. Grantham & Mark B. Cannell*

Department of Pharmacology (R-189), University of Miami School of Medicine, PO Box 016189, Miami, Florida 33101, USA

COMPELLING evidence has existed for more than a decade for a sodium/calcium (Na-Ca) exchange mechanism in the surface membrane of mammalian heart muscle cells¹⁻³ which exchanges about three sodium ions for each calcium ion⁴⁻⁶. Although it is known that cardiac muscle contraction is regulated by a transient increase in intracellular calcium ($[Ca^{2+}]_i$) triggered by the action potential⁷, the contribution of the Na-Ca exchanger to the $[Ca^{2+}]_i$ transient and to calcium extrusion during rest is unclear. To clarify these questions, changes in $[Ca^{2+}]_i$ were measured with indo-1 in single cardiac myocytes which were voltage clamped and dialysed with a physiological level of sodium. We find that Ca entry through the Na-Ca exchanger is too slow to affect markedly the rate of rise of the normal $[Ca^{2+}]_i$ transient. On repolarization, Ca extrusion by the exchanger causes $[Ca^{2+}]_i$ to decline with a time constant of 0.5 s at -80 mV. The rate of decline can be slowed e-fold with a 77-mV depolarization. Calcium extrusion by the exchanger can account for about 15% of the rate of decline of the $[Ca^{2+}]_i$ transient (the remainder being calcium resequestration by the sarcoplasmic reticulum (SR)). The ability of the cell to

* To whom correspondence should be addressed.

extrude calcium was greatly reduced on inhibiting the exchanger by removing external sodium, which itself led to an increase in resting $[Ca^{2+}]_i$. This finding is in contrast to the suggestion that calcium extrusion at rest is mediated mainly by a sarcolemmal Ca-ATPase⁸. Near equilibrium, the properties of the Na-Ca exchanger were consistent with a stoichiometry of 3Na:1Ca, and since the exchanger can operate in the absence of potassium, the cardiac Na-Ca exchanger must be different from that of rod outer segments⁹.

Indo-1, a fluorescent calcium indicator¹⁰, was introduced into single ventricular myocytes by low-resistance patch pipettes that were used to voltage clamp the cell¹¹. Using verapamil to block calcium channels and ryanodine to inhibit sarcoplasmic reticulum calcium sequestration, voltage-dependent calcium transport across the surface membrane was recorded as changes in $[Ca^{2+}]_i$. When the sodium concentration in the patch pipette was set to 8 mM, a level similar to that measured under physiological conditions^{12,13}, depolarization resulted in an increase in $[Ca^{2+}]_i$ and repolarization resulted in $[Ca^{2+}]_i$ decreasing to the pre-depolarization level (Fig. 1a). The rate of change of $[Ca^{2+}]_i$ and the final level of $[Ca^{2+}]_i$ depended approximately exponentially on membrane potential. When sodium was omitted from the pipette these changes in $[Ca^{2+}]_i$ did not occur (not shown), supporting the idea that they arise from the operation of the Na-Ca exchanger¹⁴. The exchanger tends to make the electrochemical gradient for calcium ($\Delta\tilde{\mu}_{Ca}$) equal to the sodium electrochemical gradient times the number of sodium ions exchanged per calcium ion ($\Delta\tilde{\mu}_{Ca} = n\Delta\tilde{\mu}_{Na}$) so that $\Delta\tilde{\mu}_{Ca}/F$ measured at steady state should be linearly related to the membrane potential (E_m) with a slope of n and intercept of $-nE_{Na}$

(see Fig. 1 legend). As shown in Fig. 1b, the intercept of the curve through the data (from five cells) predicts that $-nE_{Na} = -238$ mV. If the level of sodium inside the cell ($[Na^+]_i$) is the same as that in the pipette (8 mM), then E_{Na} would be ~ -76 mV which suggests that $n = 3$ (99% confidence limits: $2.9 < n < 3.3$).

The observed data points are assumed to fall below the theoretical equilibrium value for a 3Na:1Ca exchange (shown by the solid line) at very positive E_m because of calcium extrusion through a sarcolemmal Ca-ATPase ($J_{Ca-ATPase}$), and above it at negative E_m because of a calcium leak flux ($J_{Ca(leak)}$) that will increase as $\Delta\tilde{\mu}_{Ca}/F$ increases. $J_{Ca-ATPase}$ and $J_{Ca(leak)}$ will prevent the exchanger achieving equilibrium except where these fluxes are equal (and opposite) and preclude determination of n from the slope of the relationship between $\Delta\tilde{\mu}_{Ca}$ and E_m . However, the intercept of our data is close to the predicted equilibrium condition of the Na-Ca exchange (shown by the solid line in Fig. 1b), so the data is consistent with a 3Na:1Ca exchange mechanism. The deviation from linearity (principally due to $J_{Ca(leak)}$) predicts lower stoichiometries being estimated at less positive potentials, as have been observed^{13,15}. The stoichiometry that we have measured is in agreement with that obtained recently from measurements of the reversal potentials of exchanger currents⁶.

When the exchanger is at equilibrium, $J_{Ca-ATPase} = -J_{Ca(leak)}$ and this condition is predicted to occur at about -10 mV (assuming $n = 3$ and $[Na^+]_i = 8$ mM, see Fig. 1b). At more negative potentials, $J_{Ca(leak)}$ is likely to increase and this increase must be balanced by the Na-Ca exchange since $J_{Ca-ATPase}$ should decrease (the decrease in $[Ca^{2+}]_i$ and increase in $\Delta\tilde{\mu}_{Ca}$ that accompany hyperpolarization should inhibit a Ca-ATPase).

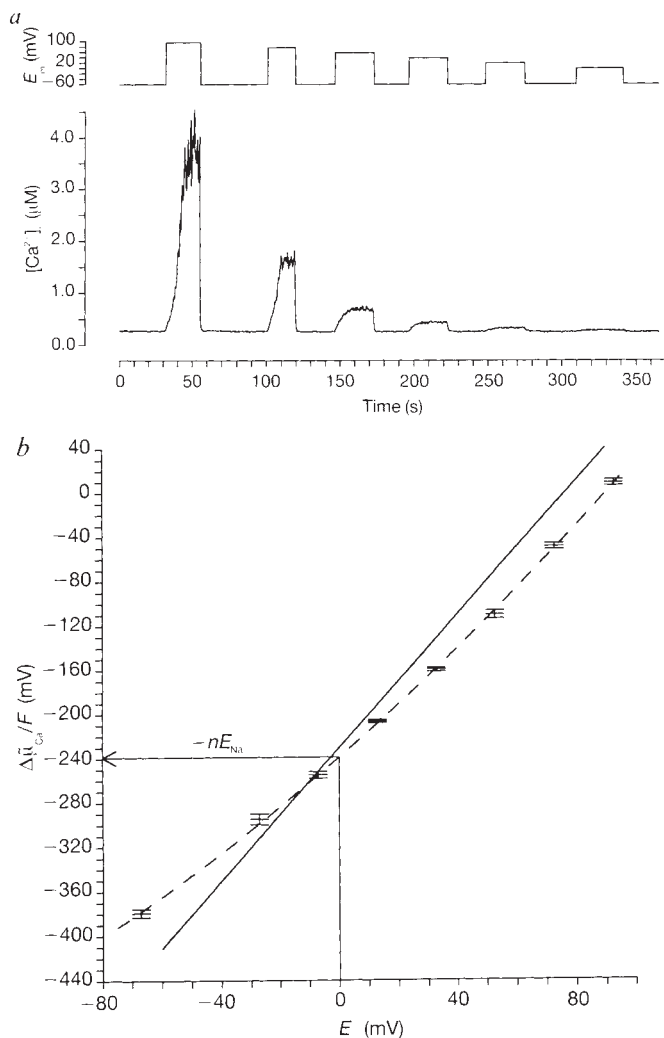


FIG. 1 a, The effect of depolarization on resting $[Ca^{2+}]_i$. Changes in $[Ca^{2+}]_i$ resulting from depolarization to various potentials in the range $+100$ to 0 mV. The pulse potentials are separated by 20 mV and the steady level of $[Ca^{2+}]_i$ reached during the pulse has a nearly exponential dependence on membrane potential. The increased noise in the $[Ca^{2+}]_i$ record at higher $[Ca^{2+}]_i$ arises from the non-linear relationship between fluorescence and $[Ca^{2+}]_i$. Note that the rate of rise of $[Ca^{2+}]_i$ is slower than the rate of decline of $[Ca^{2+}]_i$. b, Dependence of the electrochemical gradient for calcium entry ($\Delta\tilde{\mu}_{Ca}/F$) on the membrane potential (E_m). This figure shows pooled data from experiments on five different cells, the error bars correspond to ± 1 s.e.m. The dashed line is an arbitrary second-order polynomial fitted to the data to enable estimation of the y-intercept of the data, as shown. The solid line indicates the expected relationship between $\Delta\tilde{\mu}_{Ca}/F$ and E_m for a 3Na:1Ca exchange which is at equilibrium.

METHODS. The methods were similar to those published elsewhere^{11,14}. After isolation, single guinea-pig cardiac myocytes were transferred to an experimental chamber^{2,3} on the stage of a modified Nikon inverted microscope. The cells were continuously superfused at 35°C with saline containing 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM $CaEGTA$, 10 mM glucose, 10 mM HEPES, pH 7.4 . After establishing recording conditions, calcium currents were blocked with 20 μM verapamil, sodium currents with 10 μM tetrodotoxin, potassium currents with 4 mM tetraethylammonium and sarcoplasmic reticulum activity was inhibited by adding 30 μM ryanodine to the bathing solution. The patch electrode filling solution included (in mM): 135 TEA, 8 NaCl, 3.3 ATP (Mg-salt), 12 phosphocreatine (Tris-salt), creatine phosphokinase (30 U ml^{-1}), 30 PIPES, and 50 μM Indo-1 (K-salt), pH 7.2 . To obtain the data shown in Fig. 2a, verapamil was not included in the bathing solution and NaCl was omitted from the pipette filling solution. Indo-1 fluorescence was recorded at 400 - and 500 -nm wavelengths while epifluorescence illumination at 360 nm was used¹⁰. $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence, $R = (F_{400}/F_{500})$ after subtraction of cell autofluorescence and the equation $[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R)$ where R_{min} and K_d were determined in vitro^{10,24} and R_{max} determined inside the cell by electrically disrupting the sarcolemma. A computational form of $\Delta\tilde{\mu}_{Ca} = n\Delta\tilde{\mu}_{Na}$ is $2F(E_m - E_{Ca}) = nF(E_m - E_{Na})$ where E_m is the membrane potential, E_{Ca} and E_{Na} the Nernst potentials for calcium and sodium respectively, and n the stoichiometry. Thus $\Delta\tilde{\mu}_{Ca}/F = nE_m - nE_{Na}$. The Nernst potential for calcium was calculated from the external $[Ca^{2+}]$ and the dye indicated $[Ca^{2+}]_i$, assuming that the calcium activity coefficients are the same inside and outside the cell.

These considerations lead to the conclusion that the Na-Ca exchange must contribute to the regulation of resting $[Ca^{2+}]_i$ at more negative potentials. This point was examined further by removing sodium from the pipette and bathing solutions to inhibit the exchanger. When external sodium was removed (Fig. 2a) $[Ca^{2+}]_i$ slowly increased, and after $[Ca^{2+}]_i$ was increased by activating calcium channels with depolarization (note that calcium channel antagonists were not used in this experiment) the cell was unable to return $[Ca^{2+}]_i$ to resting levels. The half time of decline of $[Ca^{2+}]_i$ after the depolarizing pulse was 10 s, which is an order of magnitude slower than when the Na-Ca exchange was active (see below). The level of $[Ca^{2+}]_i$ reached during the depolarizing pulse was greater when external sodium was removed because calcium extrusion by the exchanger would have opposed the increase in $[Ca^{2+}]_i$. In agreement with these results, recent mechanical experiments have shown that the cell relaxes by less than 15% after 1 s in the absence of sodium (compared with about 83% in its presence)¹⁶. Thus under the conditions of our experiments, we find that the calcium pump extrudes only a fraction of $J_{Ca(leak)}$ at rest. This result is at variance with the view that the calcium pump sets the resting level of $[Ca^{2+}]_i$ whereas calcium extrusion by the Na-Ca exchange only becomes important when $[Ca^{2+}]_i$ is elevated⁸.

In salamander rod outer segments, the Na-Ca exchange may co-transport potassium and calcium in exchange for four sodium ions⁹. Such a mechanism could be compatible with the data shown in Fig. 1 only if the Nernst potential for potassium were close to $-E_{Na}$. As there was (essentially) no potassium in the pipette dialysis solution, such an equilibrium potential seems unlikely. Nevertheless, we examined the possibility of potassium co-transport by removing potassium from the bathing solution. In these conditions, removal of external sodium (Fig. 2b) results in a rapid increase in $[Ca^{2+}]_i$ that can be explained by the Na-Ca

exchange bringing calcium into the cell. When external sodium is replaced, there is a return of $[Ca^{2+}]_i$ to resting levels as the exchanger extrudes calcium. This result is unlike that reported for rod outer segments where removal of potassium results in almost complete inhibition of Na-Ca exchange⁹. Thus although we cannot exclude monovalent cations acting as a cofactor or catalyst for the exchanger^{17,18}, we have no evidence for potassium transport being a requirement for the Na-Ca exchange reaction.

To quantify the rate of calcium extrusion by the Na-Ca exchanger, cells were depolarized from -80 mV to $+60$ mV until $[Ca^{2+}]_i$ reached steady state, and then repolarized to various potentials to allow $[Ca^{2+}]_i$ to decrease (Fig. 3a). Analysis of these data (Fig. 3b) showed that the time course of the decline of $[Ca^{2+}]_i$ consisted of two exponential phases; the first phase was voltage independent and had a mean time constant of 0.06 s whereas the time constant of the second phase increased exponentially with voltage (Fig. 3c). The initial rapid decline in $[Ca^{2+}]_i$ may reflect some residual calcium uptake by the SR which was not blocked by ryanodine as, in the absence of ryanodine, $[Ca^{2+}]_i$ declines at a similar rate (not shown). The second slower component may reflect the activity of the Na-Ca exchanger, and this component had a time constant of 0.5 s at -80 mV which increased e-fold in 77 mV (shown by the solid line). It is notable that inward exchange currents in rod outer segments increase e-fold in 70 mV (ref. 19) and currents carried by the Na-Ca exchange in cardiac myocytes also exhibit a similar voltage dependence^{20,21}. These observations support the idea that the slower component of the decline of $[Ca^{2+}]_i$ is due to the activity of the exchanger, and our data can be used to examine the role of the exchanger in the cardiac contraction/relaxation cycle.

The rates of decline observed in Fig. 3c over the range of potentials that occur during the repolarization phase of the

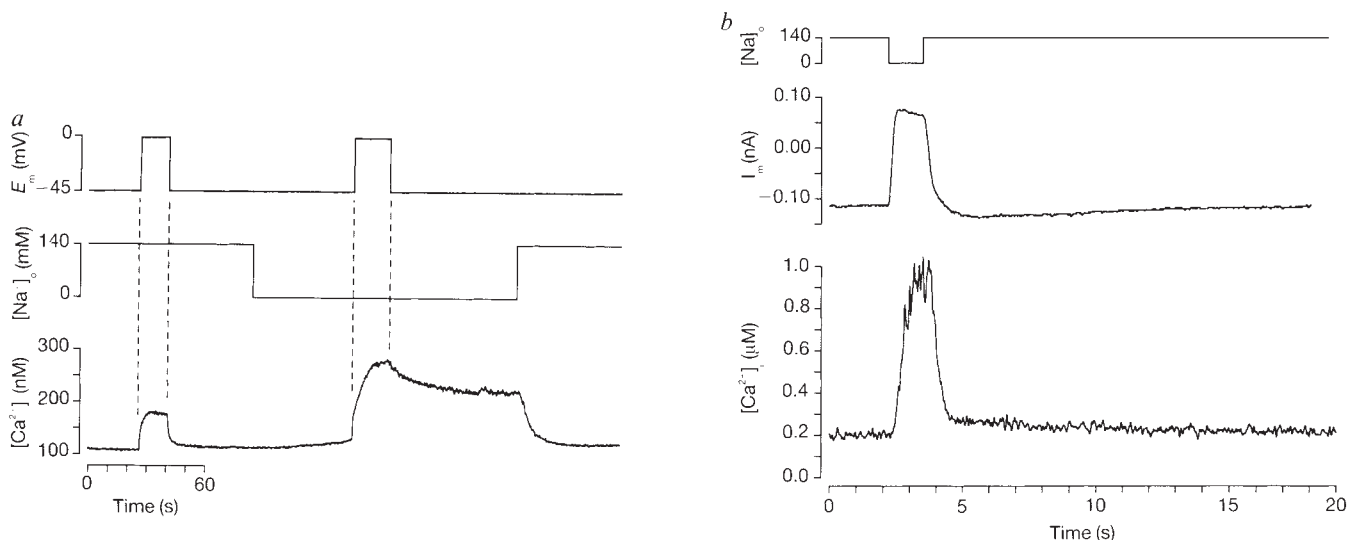


FIG. 2 Changes in $[Ca^{2+}]_i$ resulting from Na-Ca exchange inhibition. *a*, In the absence of calcium channel antagonists, depolarization resulted in $[Ca^{2+}]_i$ increasing, and this increase was rapidly reversed when the cell was repolarized in the sodium-containing bathing saline. These increases in $[Ca^{2+}]_i$ arise from the activation of calcium channels as the omission of sodium from the pipette dialysis solution prevented calcium entry through the exchanger (the effect of removing external sodium when internal sodium is present is shown in panel *b*). When sodium was removed (by isosmotic replacement with lithium) $[Ca^{2+}]_i$ slowly increased. Depolarization in the absence of sodium resulted in a greater increase in $[Ca^{2+}]_i$, and $[Ca^{2+}]_i$ slowly declined to an elevated level after repolarization. This and four other experiments showed that in the absence of sodium $[Ca^{2+}]_i$ does not decrease to the level observed in the presence of sodium. Returning sodium

to the bathing solution resulted in a rapid decrease in $[Ca^{2+}]_i$. *b*, The cell was exposed to a potassium free bathing solution for 5 min and then sodium was removed by rapidly superfusing the cell with a bathing solution in which all sodium was replaced by lithium. Removal of external sodium induces an outward current and an increase in $[Ca^{2+}]_i$. Replacement of sodium decreases $[Ca^{2+}]_i$ and induces an inward current that declines as $[Ca^{2+}]_i$ returns. The membrane potential was constant at -80 mV and $[Na^+]_i$ was 8 mM. Similar results were observed in four other experiments.

METHODS. Rapid solution changes were accomplished by moving a tube (100 μ m bore) from which a sodium-free solution was freely flowing up to the cell. The tube was then moved away from the cell to allow the control bathing solution flowing through the chamber to return. The timing of the command to change the bathing solution is indicated in each panel.

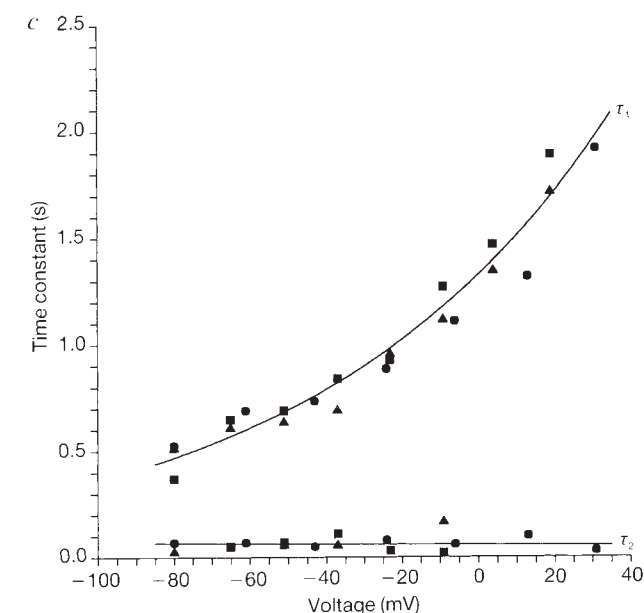
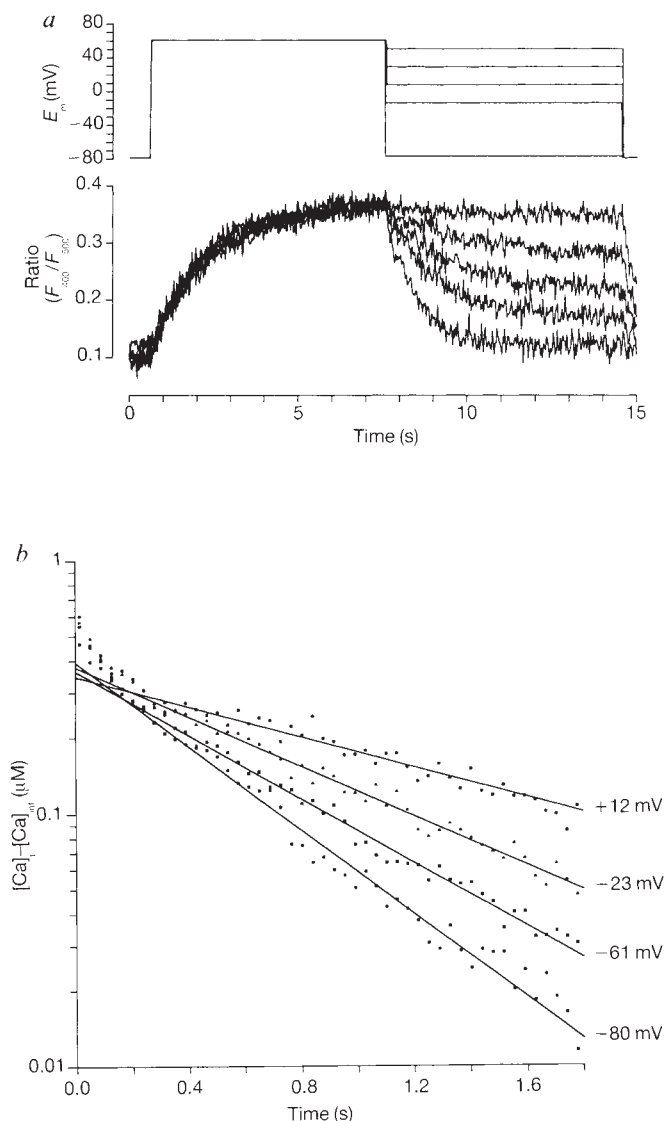


FIG. 3 Kinetics of the changes in $[Ca^{2+}]_i$ resulting from changes in membrane potential. *a*, Original experimental traces showing original records of the changes in fluorescence ratio in response to changes in E_m . E_m was stepped from -80 to +60 mV for 7 s and then changed to various potentials for a further 7 s. With depolarization, the Indo-1 fluorescence ratio increased (indicating an increase in $[Ca^{2+}]_i$) and on repolarization declined to a new level with a rate that was dependent on E_m . *b*, The ratio of fluorescence was converted to $[Ca^{2+}]_i$, and then the time course of the decline in $[Ca^{2+}]_i$ at various potentials was fitted by a non-linear least squares method to a bi-exponential decline to a constant level. For clarity, only the fit of second slower exponential component is illustrated (solid lines). The steady level of $[Ca^{2+}]_i$ ($[Ca]_{int}$) provided by the fitting program has been subtracted from the data. *c*, Summary of the analyses of data from three different cells. The time constants of the two exponential terms fitted to the data are plotted against E_m . The first rapid component (τ_1) is voltage independent while the second slower component (τ_2) is voltage dependent. The solid line shows an exponential function fitted to the data given by the equation $\tau_2 = \tau_{Em=0} \exp(E_m/k)$ (where τ_2 is the time constant in seconds, E_m in mV, $\tau_{Em=0} = 1.32$ s, $k = 77$ mV).

action potential indicate that the Na-Ca exchanger is removing calcium with a time constant between 0.5 and 1.3 s. The $[Ca^{2+}]_i$ treatment in the absence of ryanodine decreases with a time constant of about 150 ms (ref. 11) suggesting that ~15% of the rate of decline of $[Ca^{2+}]_i$ may be attributed to the Na-Ca exchange, the remainder to calcium uptake by the SR. Figure 3a indicates that the rate of rise of $[Ca^{2+}]_i$ is ~ 0.3 s $^{-1}$ at +60 mV and as the normal rate of rise of the $[Ca^{2+}]_i$ transient¹¹ is 100 s $^{-1}$, we can conclude that the exchanger supplies a very small fraction (<1%) of the total calcium needed for contraction. But if the $[Na^+]_i$ concentration is increased, the contribution of the Na-Ca exchange will increase and in connection with this point it is notable that twitches have been observed in the presence of calcium channel antagonists when $[Na^+]_i$ is increased by sodium pump inhibition²².

The data presented here support the idea that changes in action potential time course will have important effects on calcium extrusion through the Na-Ca exchange during the $[Ca^{2+}]_i$ transient¹⁴, even if calcium influx through the exchanger is relatively unimportant at normal levels of $[Na^+]_i$. In addition, it is likely that calcium extrusion by the exchanger may be more important than the calcium pump in determining $[Ca^{2+}]_i$ at rest. Thus, calcium extrusion during the $[Ca^{2+}]_i$ transient and diastolic period by the Na-Ca exchange mechanism will be an important determinant of the force of contraction of the heart. For example, activity-dependent increases in $[Na^+]_i$ will con-

tribute to the positive force-frequency relationship of the heart by decreasing calcium extrusion by the Na-Ca exchange. □

Received 20 October 1989; accepted 27 March 1990.

- Reuter, H. & Seitz, N. *J. Physiol., Lond.* **195**, 451-470 (1968).
- Glitsch, H. G., Reuter, H. & Scholz, H. *J. Physiol., Lond.* **209**, 25-43 (1970).
- Blaustein, M. P. *J. cardiovasc. Pharmac.* **12**, S56-S58 (1988).
- Pitts, B. J. R. *J. biol. Chem.* **254**, 6232-6235 (1979).
- Reeves, J. P. & Hale, C. C. *J. biol. Chem.* **259**, 7733-7739 (1984).
- Harata, T., Matsuoka, S. & Noma, A. *J. Physiol., Lond.* **410**, 227-249 (1989).
- Chapman, R. A. *Am. J. Physiol.* **245**, H535-H552 (1983).
- Carnoni, P. & Carafoli, E. *J. biol. Chem.* **256**, 3263-3270 (1981).
- Cervetto, L., Lagnado, L., Perry, R. J., Robinson, D. W. & McNaughton, D. A. *Nature* **337**, 740-743 (1989).
- Grynkiewicz, G., Poenie, M. & Tsien, R. Y. *J. biol. Chem.* **260**, 3440-3450 (1985).
- Cannell, M. B., Berlin, J. R. & Lederer, W. J. *Science* **238**, 1419-1423 (1987).
- Ellis, D. *J. Physiol., Lond.* **273**, 211-240 (1977).
- Sheu, S. S. & Fozzard, H. A. *J. gen. Physiol.* **80**, 325-351 (1982).
- Barcelinas-Ruiz, L., Beuckelmann, D. J. & Wier, W. G. *Science* **238**, 1720-1722 (1987).
- Bers, D. M. & Ellis, D. *Pflügers Arch. ges. Physiol.* **393**, 171-178 (1982).
- Bridge, J. H. B., Spitzer, K. W. & Ershler, P. R. *Science* **241**, 823-825 (1988).
- Baker, P. F., Blaustein, M. P., Hodgkin, A. L. & Steinhardt, R. A. *J. Physiol., Lond.* **200**, 431-458 (1969).
- Gadsby, D. C., Nakao, M., Noda, M. & Shepherd, R. N. *J. Physiol., Lond.* **407**, 135P (1988).
- Lagnado, L., Cervetto, L., McNaughton, P. A. *Proc. natn. Acad. Sci. U.S.A.* **85**, 4548-4552 (1988).
- Hume, J. R. & Uehara, A. *J. gen. Physiol.* **87**, 857-884 (1986).
- Kimura, J., Miyamae, S. & Noma, A. *J. Physiol., Lond.* **384**, 199-222 (1987).
- Berlin, J. R., Cannell, M. B. & Lederer, W. J. *Am. J. Physiol.* **253**, H1540-H1547 (1987).
- Cannell, M. B. & Lederer, W. J. *Pflügers Arch. ges. Physiol.* **406**, 536-539 (1986).
- Williams, D. A., Fogarty, K. E., Tsien, R. Y. & Fay, F. S. *Nature* **318**, 558-561 (1985).

ACKNOWLEDGEMENTS. This work was supported by grants from the NIH and the American Heart Association. We thank W. Nonner and K. Magleby for comments on the manuscript and C. Ferrandelli for assistance in manuscript preparation.

Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160

Phillip W. Berman*, Timothy J. Gregory†, Lavon Riddle†, Gerald R. Nakamura*, Mark A. Champe*, James P. Porter‡, Florian M. Wurm†, Robert D. Hersherg†, E. Kathy Cobb§ & Jorg W. Eichberg§

* Departments of Immunobiology, † Process Sciences, and ‡ Medicinal and Analytical Chemistry, Genentech, Inc., South San Francisco, California 94080, USA

§ Department of Virology and Immunology, Southwest Foundation for Biomedical Research, San Antonio, Texas, USA

THE development of a vaccine to provide protective immunity to human immunodeficiency virus type 1 (HIV-1), the virus causing AIDS, would be the most practical method to control its spread. Subunit vaccines consisting of virus envelope glycoproteins, produced by recombinant DNA technology, are effective in preventing viral infections¹. We have now used this approach in the development of a candidate AIDS vaccine. Chimpanzees were immunized with recombinant forms of the HIV-1 glycoproteins gp120 and gp160 produced in Chinese hamster ovary cells, and then challenged with HIV-1. The control and the two animals immunized with the gp160 variant became infected within 7 weeks of challenge. The two animals immunized with the gp120 variant have shown no signs of infection after more than 6 months. These studies demonstrate that recombinant gp120, formulated in an adjuvant approved for human use, can elicit protective immunity against a homologous strain of HIV-1.

The structure, expression and immunogenicity of recombinant gp120 (rgp120) and recombinant soluble gp160 (rsgp160) have been described previously^{2,3}. A diagram and description of each molecule are in Fig. 1a. Both proteins are glycosylated in a manner similar to authentic viral gp120 and bind to CD4, the cellular receptor for HIV-1, with high affinity³⁻⁵. We immunized two chimpanzees with the rsgp160 protein (relative molecular mass 140,000–150,000 (140K–150K)) and two chimpanzees with the 120K–130K rgp120 protein (Fig. 1b). A control animal was immunized with herpes simplex virus type-2 glycoprotein D (ref. 6). All three immunogens were prepared in an aluminum hydroxide adjuvant⁷ and injected according to an immunization protocol (0, 1 and 8 months) for primates⁸.

Antibodies against rgp120 were detected by radioimmunoassay only after the second immunization (4 weeks), but did not neutralize HIV-1 infectivity *in vitro*⁹ (Fig. 2a, b). A third immunization at 32 weeks resulted in a marked increase in antibody titres (Fig. 2a) and neutralizing activity. The peak neutralizing titres (1:320–1:640) were similar for both immunogens, and were far greater than those (1:5) observed for rgp120 in our previous study⁷. Immunoblot analysis (Fig. 3) of the sera collected 3 weeks after the third immunization (week 35) showed that the animals that received rgp120 possessed antibodies strongly reactive with authentic gp120 and weakly reactive with gp160. The animals immunized with rgp160 formed antibodies that reacted with viral gp160 and gp41 as well as gp120. Cellular immunity, as indicated by the ability of rgp120 to stimulate lymphocyte proliferation, could be detected in the animals immunized with rgp120 and rsgp160 after the final boost (Table 1).

On the basis of significant levels of neutralizing antibodies, we proceeded with a virus challenge at week 35 using about 10 chimpanzee-infectious-doses¹⁰ (CID₅₀) of the IIIB isolate of

TABLE 1 Immunological and virological characteristics of chimpanzees before and after HIV-1 infection

Animal number	Control x246	rsgp160- immunized x247 x261		rgp120- immunized x262 x265	
At time of virus challenge (35 weeks)					
Anti-gp120 titre	—	4.3	4.3	4.8	3.9
Antibodies to gp41	—	+	+	—	—
Proliferation to rgp120	—	+	+	+	+
Neutralizing titre	<10	160	160	320	640
CD4-blocking antibodies	<1	2.2	2.5	2.5	1.7
Anti-PND titre	<10	145	149	491	844
After HIV-1 challenge					
First co-cultivation of HIV-1 (wk)	6	6	7	—	—
Seroconversion to p24 (wk)	11	5	5	—	—
Seroconversion to p17 (wk)	13	5	5	—	—
PCR (53 wk)	+	+	+	—	—
Neutralizing titre (60 wk)	10	80	80	<10	<10
Proliferation to rgp120 (60 wk)	—	+	+	—	—
Anti-gp120 titre (63 wk)	—	3.2	2.9	—	—

Antibody titres against gp120 were determined in a liquid-phase radioimmunoassay; the data presented represent log values from end-point dilution titrations (legend to Fig. 2a). The presence of antibodies to gp41 and the time taken for antibodies to p24 and p17 to appear were determined by immunoblot analysis of sequential bleeds using commercial (Dupont and Biorad) western blot strips. The co-cultivation of HIV-1 and the proliferation of chimpanzee lymphocytes to rgp120 was as described previously⁸. *In vitro* neutralizing antibodies were measured by the method of Robertson *et al.*¹⁰ (see also legend to Fig. 4). The ability of antibodies to rgp120 or rsgp160 to inhibit the binding of rgp120 to CD4 was measured in an enzyme-linked immunosorbent assay in which wells in a microtitre plate were coated with rgp120. Test-antibodies were serially diluted and incubated with the rgp120 'capture' plate for 1 h. After washing three times in PBS, 0.05% Tween-20, soluble CD4 (rCD4) was added to the plate and incubated for 2 h with gentle shaking. The wells were aspirated, washed five times, then incubated with a horseradish peroxidase-conjugated monoclonal antibody to CD4. After washing, substrate was added and colour development proceeded for 30 min until stopped by the addition of 1 M HCl. The absorbance in each well was determined at a wavelength of 450 nm in a plate-reading spectrophotometer. The data are reported as the log of the serum dilution required to inhibit binding by 50%. Anti-PND titres were determined in an enzyme-linked immunosorbent assay as described in Fig. 2c. Polymerase chain reaction (PCR) reactivity was determined using the method of Kellogg and Kwok¹³. In brief, frozen lymphocytes were processed in PCR lysis buffer and the DNA was phenol-chloroform extracted and ethanol precipitated. A sample of the DNA (equivalent to the amount in 150,000–300,000 cells) was subjected to 40 cycles of PCR using the SK68 and SK69 primers. Samples were adjusted to 25 mM NaCl and hybridized in solution for 30 min at 55 °C to 0.5 pmol of primer SK70 end-labelled with [γ -³²P]ATP. Samples were resolved on a 10% acrylamide mini-gel in TBE buffer. The gel was then dried and used to expose an X-ray film. Positive control DNA was prepared from a transfected Chinese hamster ovary (CHO) cell line expressing gp160. DNA samples were tested for their ability to be amplified using probes to the β -globin gene. Samples were considered positive for HIV-1 if the labelled primer hybridized to a band of 141 base pairs.

HIV-1 (see legend to Fig. 2a). The challenge stock was prepared from a standard inoculum that has been titrated in chimpanzees and used successfully for other chimpanzee-challenge studies¹⁰⁻¹². The same dose of virus (10 CID₅₀) from this stock has successfully infected three additional chimpanzees in three independent studies (J.W.E., unpublished data). After virus challenge, the magnitude and the duration of the antibody response in the rgp120-immunized animals differed markedly from that of the control and the rsgp160-immunized animals. The anti-gp120 antibody titre in these animals progressively declined with time and eventually reached baseline values (Table

1, Fig. 2a). By contrast, the titre of anti-gp120 antibodies in the rsgp160 animals declined for only 7–8 weeks after virus challenge, then increased to a level higher than the peak achieved by hyperimmunization and was then sustained at an intermediate level. These results suggest that the rgp120-immunized animals were protected from HIV-1 infection, whereas the rgp160-immunized animals developed an infection sufficient to stimulate an anamnestic immune response. Antibodies against gp120 were detected in the control animal by radioimmunoassay at 16 weeks post-challenge, indicating that this animal had also become infected with HIV-1.

Evidence of HIV-1 infection, as indicated by the formation of antibodies against the HIV-1 core proteins, was first detected in the two rsgp160-immunized animals at 5 weeks post-challenge as a faint p24 band in the immunoblot assay (data not shown). Antibodies to p17 and p55 became apparent at later dates (Table 1 and Fig. 3). Antibodies to p24 were first detected in the control animal at 11 weeks post-challenge (data not shown), and reactivity with p17, p55 and gp120 could be detected at later dates (Table 1 and Fig. 3). Interestingly, the intensity of the antibody response to HIV-1 structural proteins (for example, p55, p24 and p17) was far greater throughout the study in the rsgp160-immunized animals (Fig. 3) than in the control. Antibodies to gp120 were present in the rgp120-immunized animals at the time of challenge, but had largely disappeared by 13 weeks post-challenge (Fig. 3). The animals immunized with rgp120 have not so far (26 weeks post-challenge) seroconverted to any of the HIV-1-encoded proteins other than gp120 and gp160, suggesting that they were protected from HIV-1 infection.

Virus co-cultivation studies were carried out to determine whether viable virus could be recovered from any of the immunized animals. HIV-1 could be recovered from the control and the rsgp160-immunized animals at 6–7 weeks post challenge (Table 1) but has not yet been recovered from either of the rgp120-immunized animals. Further evidence of HIV-1 infection in the control and the two rsgp160-immunized animals, but not the rgp120-immunized animals, was provided by polymerase-chain-reaction analysis at 18 weeks post-challenge (Table 1).

Changes in the titres of neutralizing antibodies after HIV-1 challenge paralleled those seen in the anti-gp120 titres (Fig. 2b). The neutralizing titres in the rgp120-immunized animals were maximal at the time of virus challenge and then steadily declined to the level of preimmune sera by 60 weeks. The neutralizing titres in both of the rsgp160-immunized animals were lower than their peak values at the time of challenge (Table 1 and Fig. 2b). The levels declined further for several weeks but sharply increased at 7 weeks post-challenge in response to the production of viral proteins. Neutralizing antibodies were not detected in the control until 17 weeks post-challenge. Because the levels of neutralizing antibodies present in chimpanzees immunized with rgp120 and rsgp160 were essentially identical over the 4-week period that followed the last boost, it was surprising that only the rgp120-immunized animals were protected from HIV-1 infection.

One possible explanation for the difference in protection between the two groups of animals is, that on the actual day of virus challenge, the rsgp160-immunized animals had slightly lower neutralization titres (that is, 1:160) than the rgp120-immunized animals (1:320 and 1:640). If the level of antibody present on the actual day of challenge is crucial, then our data indicate that titres >1:160 are required to prevent infection. But other factors, such as differences in the concentration of antibodies against particular epitopes, could provide an alternative explanation. One specificity examined was the level of antibodies able to inhibit the binding of gp120 to CD4. Previously^{3,4} we noted that both rgp120 and rsgp160 are effective in eliciting antibodies that disrupt this interaction. Analysis of sera at the time of challenge showed that the animals immunized with rgp120 and rsgp160 all possessed antibodies that blocked binding to CD4, but that the blocking titre did not correlate

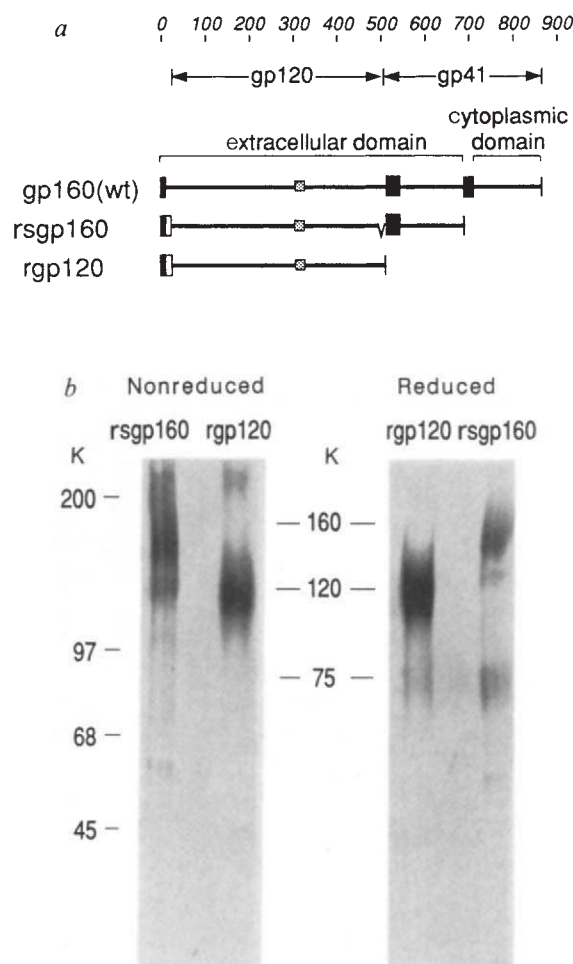


FIG. 1 Composition of rgp120 and rsgp160 immunogens. *a*, Schematic representation of the HIV-1 glycoprotein precursor, gp160, and the two variant gp160-derived proteins, rgp120 and rsgp160, used in the present study. Numbers at the top refer to amino-acid positions. Both molecules were produced in CHO cell lines under transcriptional control of the SV40 early promoter as described previously^{2,3}. Both proteins differ from wild-type (wt) gp160 at the N terminus. In the rgp120 protein, the signal sequence and 31 residues of gp160 have been replaced with the signal sequence and 25 amino acids from the mature N terminus of herpes simplex virus type 1 glycoprotein D (HSV-1 gD)³. In the rsgp160 protein, the signal sequence and 12 amino acids of gp160 have been replaced with the signal sequence and nine amino acids from the mature N terminus of HSV-1 gD. In addition, rsgp160 differs from the viral gp160 in that the transmembrane domain and cytoplasmic tail (amino acids 684–856) and the gp120–gp41 proteolytic processing site (amino acids 502–511) have been deleted by *in vitro* mutagenesis as described previously⁴. The location of the hydrophobic regions corresponding to the signal sequence (N terminus of gp120), the fusion domain (N terminus of gp41) and the transmembrane domain (middle of gp41) are indicated by black boxes. The N-terminal sequences of HSV-1 gD are indicated by open boxes and the principal neutralizing determinant (PND) is shown by stippled boxes. *b*, Silver-stained SDS-PAGE of purified rgp120 and rsgp160 under reducing and nonreducing conditions. Both recombinant glycoproteins were purified from growth-conditioned cell culture supernatants by immunoaffinity chromatography and gel-permeation chromatography to greater than 99% and 95% purity for rgp120 and rsgp160, respectively. Under nonreducing conditions the rgp120 contained less than 5% dimer and the rsgp160 was about 50% dimer and higher-order oligomers. Both proteins are endoproteolytically cleaved between Arg 315 and Ala 316 in the V3 region of the gp120 portion of each molecule. In rgp120 this results in an N-terminal 75K fragment and a 50K C-terminal fragment, whereas in rsgp160 the same cleavage results in two fragments both of ~75K. On SDS-polyacrylamide gels the fragments are visible only under reducing conditions because of disulphide bonding between cysteine residues 296 and 331 (ref. 6). The rgp120 preparation contained less than 5% of the cleaved form whereas ~40% of the rsgp160 was cleaved. *M_r* calibration is shown (in thousands) on the left of each gel.

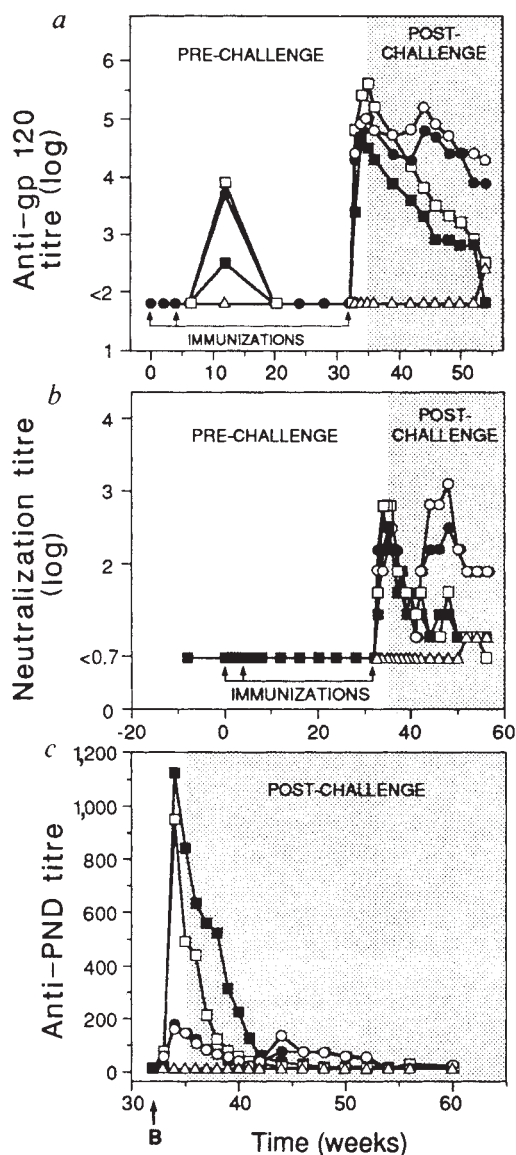


FIG. 3 Immunoblot analysis of sera from animals immunized with candidate HIV-1 vaccines. Sera from chimpanzees immunized with rsgp160 (x247, x261), rgp120 (x262, x265) or HSV-1 gD (x246) were diluted and incubated with commercial (Dupont) immunoblot strips. The strips were incubated with alkaline phosphatase-coupled goat anti-human IgG (Cappel) and developed with a phosphatase substrate system (Kirkegaard and Perry Laboratories). The data shown represent results obtained on the same day using the same assay strips. Week 35 represents the time of challenge. The positive control consisted of serum from an HIV-1-infected individual.

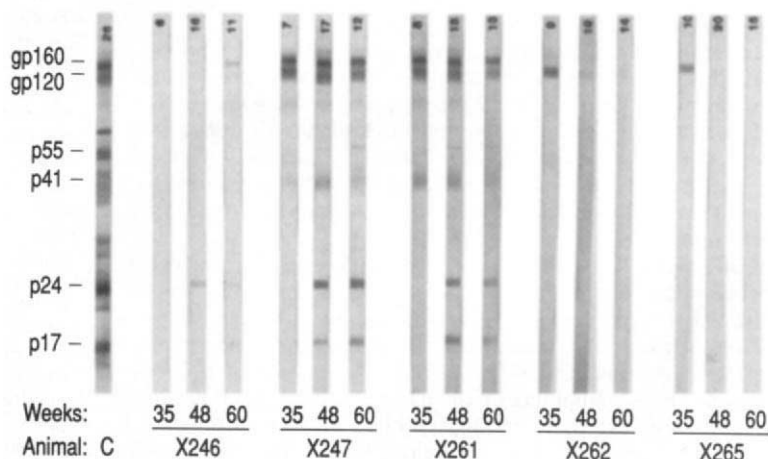


FIG. 2 Characteristics of antibodies to rgp120 and rsgp160 in animals immunized with candidate HIV-1 vaccines. Chimpanzees were immunized with 300 μ g per dose of rgp160 (x247 and x261), rgp120 (x262 and x265) or herpes simplex virus glycoprotein D (x246), at 0, 4 and 32 weeks (arrows). All immunogens were incorporated in aluminum hydroxide adjuvant containing 2 mg equivalents of Al^{3+} per mg of protein. Blood was taken at the time points indicated, and the sera analysed for antibodies to rgp120 or rsgp160 in the assays described below. All animals were challenged by intravenous injection of 10 $TCID_{50}$ units (40 $TCID_{50}$ units) of the IIIB isolate of HIV-1 at 35 weeks. The challenge stock was provided by Dr L. O. Arthur (NCI) and had been extensively titrated in chimpanzees¹⁰. This stock has been used successfully to infect more than 15 chimpanzees at the Southwest Foundation for Biomedical Research (J.W.E., unpublished data). $\square$ and $\blacksquare$, rgp120-immunized animals, x262 and x265, respectively; $\circ$ and $\bullet$, rsgp160-immunized animals, x247 and x261, respectively; $\triangle$, the control animal, x246. **a**, Antibodies to gp120 detected by immunoprecipitation of ^{125}I -labelled gp120 in a liquid-phase radioimmunoassay described previously⁷. **b**, The *in vitro* neutralizing activity in sera from chimpanzees immunized with rgp120 and rsgp160, determined by a neutralization assay similar to that described by Robertson *et al.*⁹. In brief, diluted samples of serum were incubated with 100 $TCID_{50}$ units of virus (IIIB isolate) for 60 min at 20 $^{\circ}C$. The mixture was transferred to cell-culture plates containing 5×10^4 MT4 cells and incubated for 7 days. Viral lysis of infected cells was detected through the use of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) as a vital stain. Neutralization assays were performed in duplicate. Variation between replicates was less than one dilution (two-fold). Sera from HIV-1 infected and uninfected chimpanzees were used as positive and negative controls, and gave neutralizing titres of 1:640 and $<1:10$, respectively. **c**, Relative concentration of antibodies reactive with the PND¹⁶ was determined in an enzyme-linked immunosorbent assay where a synthetic peptide sequence NNRKSIIRIQRGPGRAFTIGKIG (single-letter amino-acid code) corresponding to amino-acid residues 301–324 of gp120 from the IIIB isolate, was used to coat microtitre dishes at a concentration of 2 μ g ml^{-1} . After an overnight incubation at 4 $^{\circ}C$, the coated wells were washed with PBS containing 0.05% Tween-20 and treated with a blocking buffer of 0.8% BSA in PBS. Each sample of chimpanzee serum was serially diluted over a range of 1:10 to 1:10,240 and 100 μ l was incubated in the wells for 1.5 h at room temperature. The wells were then washed four times with PBS containing 0.05% Triton X-100. Labelled horseradish peroxidase-conjugated goat anti-human IgG was incubated for 1 h and the plates washed four times with PBS, 0.05% Triton X-100. Antibody binding was indicated by a change in the colour after the substrate o-phenylenediamine dihydrochloride was added. The colorimetric reaction was stopped by the addition of 2.5 M H_2SO_4 , and the absorbance at wavelength of 492 nm was measured in a plate-reading spectrophotometer.

either with protection from infection or with neutralizing titre (Table 1).

We next considered whether a subpopulation of neutralizing antibodies might account for the difference in protection between animals treated with rgp120 or rsgp160. To test this hypothesis, we assayed the sera by enzyme-linked immunosor-

bent assay for antibodies reactive with a synthetic peptide corresponding to a region in the third hypervariable domain of gp120 that contains the principal type-specific neutralizing determinant (PND)¹⁴. When the sera from the animals immunized with rgp120 and rsgp160 were compared, we found that the 32-week immunization induced a five- to sixfold greater response to this

epitope in the animals immunized with rgp120 compared with those immunized with rsgp160 (Table 1 and Fig. 2c). Interestingly, animal x265, which had the lowest anti-gp120 titre as determined by radioimmunoassay, had the highest titre to the PND at the time of challenge, and was protected from infection.

Although this correlation is interesting, it does not prove that the lack of protection in the rsgp160-immunized animals resulted from a low level of antibodies to the PND. It is possible that the neutralizing antibodies elicited by rsgp160 are protective, but that a second factor abrogates their protective effect. Antibodies that interfere with neutralizing antibodies (for example, blocking antibodies) have been described for other retroviruses¹⁵ and there have been several reports of antibodies to HIV-1 that enhance rather than inhibit viral infection *in vitro*^{16,17}. An epitope that was present on gp41 but not gp120, and which enhanced infectivity¹⁸, could explain the difference in protection and the serological data presented in Table 1 and Fig. 3. A similarly enhanced serological response to HIV-1 core antigens was previously observed in chimpanzees immunized with a recombinant vaccinia virus expressing HIV-1 gp160 (J.W.E., unpublished data). Another alternative explanation is that protection correlates with cellular immunity (for example, cytotoxic T cells) and not neutralizing antibodies. Although this cannot be ruled out, the fact that subunit vaccines are thought to be poor inducers of cytotoxic cells restricted by major histocompatibility complex class I antigens¹⁹ argues against this possibility.

This paper represents the first report, to our knowledge, of a candidate AIDS vaccine that has succeeded in protecting chimpanzees from HIV-1 infection, and demonstrates that protection against HIV-1 does not require proteins other than gp120 or cellular immunity of a type unique to live attenuated viral vaccines. These results raise a new set of problems that need to be addressed so that the development of an HIV-1 vaccine for humans can proceed. Most importantly, it will be necessary to determine whether gp120 from the IIIB isolate of HIV-1 alone, or in combination with gp120 from other isolates, will provide protection against the great diversity of HIV-1 variants that have infected the human population. Also, we need a better understanding of the mechanism(s) of protective immunity. Finally, we need to explore the duration of the protective immunity that has been achieved with this vaccine. For a vaccine to be practical in humans, it should provide protection long after vaccination. Although these problems could complicate the development of a useful vaccine, the data reported here demonstrate that it is possible to elicit a protective immune response against HIV-1. □

Prevention of HIV-1 glycoprotein transport by soluble CD4 retained in the endoplasmic reticulum

Linda Buonocore & John K. Rose*

Departments of Pathology and Cell Biology, Yale University School of Medicine, 310 Cedar Street, New Haven, Connecticut 06510, USA

THE envelope glycoprotein (gp120/41) of the human immunodeficiency virus (HIV-1)^{1,2} attaches the virus to the cellular CD4 receptor and mediates virus entry into the cytoplasm³⁻⁵. In addition to being required for formation of infectious HIV, expression of gp120/41 at the plasma membrane causes the cytopathic fusion of cells carrying the CD4 antigen⁶⁻⁸. The expression of gp120/41 is therefore an ideal target for therapeutic strategies designed to combat AIDS. Here we show that expression of a soluble CD4 molecule, mutated to contain a specific retention signal for the endoplasmic reticulum, blocks secretion of gp120 and surface expression of gp120/41, but does not interfere with transport of wild-type CD4. By blocking transport of the HIV glycoprotein, this retained CD4 molecule prevents the fusion of CD4 cells that is normally caused by the HIV glycoprotein. Expression of the retained CD4 molecule in human T cells might therefore be useful in the intracellular immunization procedure suggested by Baltimore⁹.

Infection of T cells with HIV results in decreased membrane expression of CD4. This 'down-regulation' may result in part from intracellular binding of the HIV envelope protein precursor, gp160, to CD4 (refs 10, 11). Because only 5-15% of the total gp160 synthesized ever reaches the plasma membrane¹², transport of any CD4 bound to gp160 may be largely blocked. Our own studies (B. Crise, L.B. and J.K.R., unpublished data) have shown that gp160-CD4 complexes form efficiently in the endoplasmic reticulum (ER) and that 85-90% of CD4 transport from the ER can be blocked by coexpression of gp160. Given the efficient binding of gp160 and CD4 in the ER, we reasoned that expression of a CD4 molecule permanently retained in the ER might block surface expression of the small fraction of gp160 that is normally transported to the cell surface.

The sequence Lys-Asp-Glu-Leu (KDEL in single-letter code) is a signal that retains soluble proteins within the lumen of the ER and prevents their secretion¹³. To determine if the KDEL signal would retain soluble CD4, we first mutated the CD4 complementary DNA clone⁴ to encode soluble CD4 lacking transmembrane and cytoplasmic domains (sCD4), or soluble CD4 with the sequence Ser-Glu-Lys-Asp-Glu-Leu (SEKDEL) appended to its C terminus (sCD4-KDEL; Fig. 1). These constructs were placed under control of the bacteriophage T7 promoter so that they could be expressed in HeLa cells infected with vTF-7, a vaccinia virus that expresses the T7 RNA polymerase¹⁴. HeLa cells expressing CD4, sCD4 or sCD4-KDEL were pulse-labelled and immunoprecipitates prepared with an anti-CD4 antibody before or after a 2-h chase period. Immediately after the pulse period, all of the proteins were cell-associated (Fig. 2a). After the chase period, both CD4 and sCD4-KDEL remained fully cell-associated, whereas 50% of the sCD4 was secreted into the medium. The larger size and heterogeneity of the sCD4 in the medium results from oligosaccharide processing. We observed no secretion of sCD4-KDEL even after a 4-h chase period, indicating that the retention signal was working effectively.

To confirm that sCD4-KDEL was retained in the ER, we examined the processing of its two N-linked oligosaccharides. CD4 and sCD4-KDEL had unprocessed oligosaccharides that

Received 3 April; accepted 24 May 1990.

1. Scolnick, E. M. *et al.* *J. Am. med. Assoc.* **251**, 2812-2815 (1984).
2. Lasky, L. A. *et al.* *Science* **233**, 209-211 (1986).
3. Berman, P. W. *et al.* *J. Virol.* **63**, 3489-3498 (1989).
4. Lasky, L. A. *et al.* *Cell* **50**, 975-985 (1987).
5. Leonard, C. K. *et al.* *J. Biol. Chem.* (in the press).
6. Berman, P. W., Gregory, T., Crase, D. & Lasky, L. A. *Science* **277**, 1490-1492 (1985).
7. Berman, P. W. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **85**, 5200-5204 (1988).
8. Anderson, K. P. *et al.* *J. infect. Diseases* **160**, 960-969 (1989).
9. Robertson, G. A., Kostek, B. M., Schleif, W. A., Lewis, J. A. & Emini, E. A. *J. virol. Meth.* **20**, 195-202 (1988).
10. Arthur, L. O. *J. Virol.* **63**, 5046-5053 (1989).
11. Prince, A. M. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **85**, 6944-6948 (1988).
12. Emini, E. A. *et al.* *J. Virol.* (in the press).
13. Kellog, D. E. & Kwok, S. in *PCR Protocols* (ed. Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, J.) 337-347 (Academic, New York, 1990).
14. Rusche, J. R. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **85**, 3198-3202 (1988).
15. Massey, R. J. & Schochetman, G. *Science* **213**, 447-449 (1981).
16. Homsy, J., Meyer, M., Tateno, M., Clarkson, S. & Levy, J. A. *Science* **244**, 1357-1359 (1989).
17. Robinson, E. W. Jr, Montefiori, D. C. & Mitchell, W. M. *Lancet* **i**, 790-794 (1988).
18. Robinson, E. W. Jr *et al.* *Proc. natn. Acad. Sci. U.S.A.* **87**, 3185-3189 (1990).
19. Germain, R. N. *Nature* **322**, 687-689 (1986).

ACKNOWLEDGEMENTS. We thank D. Capon for providing the gene encoding the HIV-1 envelope glycoprotein, A. D. Levinson for the vector used to express rgp120 and rsgp160, L. A. Lasky and C. Fennie for constructing the cell line used to produce rgp120, C. Lucas for development of the rgp120 RIA and J. Burnier for providing synthetic PND peptide. We also thank R. D. Lee for veterinary assistance, P. A. Tuck, S. R. Rouse, G. I. Melendez and P. Piazza for technical assistance in virological and immunological assays, and D. Pineda, T. Fleming and J. Nobles for collection of samples. We also thank J. Objeski and R. Ward for critically reviewing this manuscript, and R. Gomez, W. Young, and H. Niall for their support in the completion of this project.

* To whom correspondence should be addressed.

were completely sensitive to endoglycosidase H (endo H)¹⁵ digestion immediately after synthesis (Fig. 2b). One of the oligosaccharides on CD4 was 80% resistant to endo H after 2 h, indicating that it had been transported through the Golgi apparatus^{16,17}, whereas sCD4-KDEL remained fully sensitive, as expected for a protein retained in the ER. Localization of sCD4-KDEL by indirect immunofluorescence also indicated retention in the ER, whereas CD4 was transported to the cell surface (data not shown).

We next coexpressed sCD4-KDEL and HIV-1 gp120 to determine if secretion of gp120 would be inhibited by sCD4-KDEL. The gp120 coding sequence (with a stop codon preceding the gp41 coding sequence) was placed in a plasmid under control of the T7 promoter and expressed in HeLa cells, either alone or with CD4, sCD4 or sCD4-KDEL. HeLa cells expressing these proteins were pulse-labelled and gp120 immunoprecipitates prepared immediately after the pulse or after a 2-h chase period. The results (Fig. 3a) showed that gp120 was secreted from cells when expressed alone, or when expressed with sCD4 or CD4. By contrast, gp120 was not secreted from cells expressing sCD4-KDEL. (Soluble gp120 expressed alone is transported more efficiently than gp160. CD4-gp120 or sCD4-gp120 complexes are not usually retained inside the cell like gp160-CD4 complexes; B. Crise, L.B. and J.K.R., unpublished data.) In each case the levels of expressed CD4, sCD4 or sCD4-KDEL were very similar, as determined by precipitation of aliquots of each lysate with anti-CD4 antibody (not shown). Although candidate bands of CD4, sCD4 or sCD4-KDEL were seen co-precipitating with gp120 in the experiment of Fig. 3, it was not possible to identify these unambiguously because of the presence of background bands in immunoprecipitates of cell lysates. In the one case where sCD4 should be present in the medium in a complex with gp120 (2 h, with sCD4), the sCD4 protein is clearly visible (arrow).

We also examined the effect of sCD4-KDEL expression on secretion of gp120 derived from gp160 by cleavage. When the entire gp160 coding region was expressed, a major protein band of relative molecular mass 160,000 (M_r 160K) was precipitated from cell lysates by anti-gp120 serum (Fig. 3b). A small fraction of pulse-labelled gp160 was converted to gp120 after 4 h and was released into the medium, presumably because it was shed

from a gp120/41 complex at the cell surface. This release of cleaved gp120 was prevented by coexpression of sCD4-KDEL, but not by sCD4.

Although CD4 is not known to be an oligomer, we were concerned that expression of sCD4-KDEL might interfere with transport of wild-type CD4 through formation of hetero-oligomeric complexes. To test this possibility, we expressed CD4 in the presence of a threefold excess of sCD4-KDEL and followed oligosaccharide processing on both molecules. Eighty per cent of the oligosaccharides at one site on CD4 were pro-

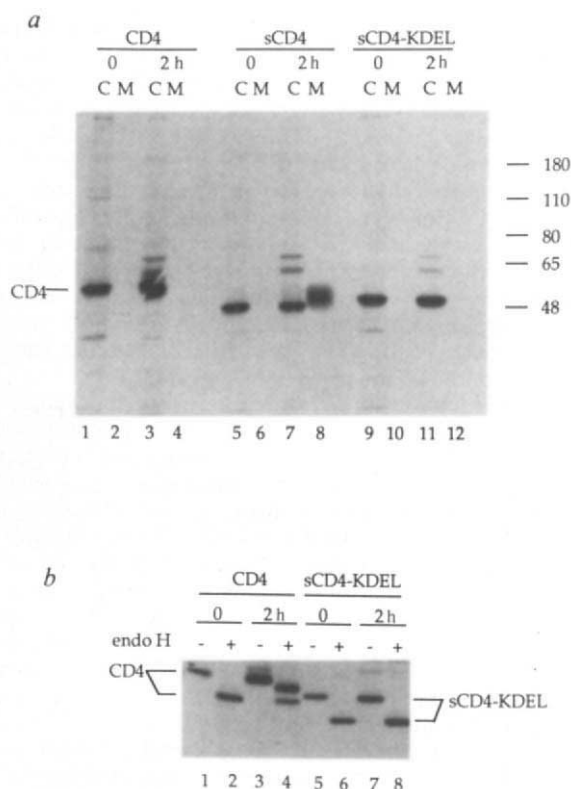


FIG. 2 sCD4-KDEL is retained in cells and contains unprocessed oligosaccharides. *a*, Autoradiogram of a polyacrylamide gel showing that sCD4 is secreted while CD4 and sCD4-KDEL remain cell-associated. HeLa cells were infected with vTF-7 and transfected with 5 μ g DNA pBS-CD4, pBS-sCD4 or pBS-sCD4-KDEL as indicated. Cells were pulse-labelled with [³⁵S]methionine and immunoprecipitates were prepared²⁷ from cell lysates (C) or medium (M) with OKT4 antibody before or after a 2-h chase period. Protein samples were resolved by electrophoresis on a 10% polyacrylamide gel and visualized by autoradiography. Positions of markers indicating M_r ($\times 10^{-3}$) are shown. *b*, sCD4-KDEL retains endo H-sensitive oligosaccharides. HeLa cells were infected with vTF-7 and transfected with 5 μ g pBS-CD4 or pBS-sCD4-KDEL as indicated. After pulse labelling, immunoprecipitates were prepared from cell lysates before or after a 2-h chase period. Immunoprecipitates were divided in half and either mock-treated (–) or treated (+) with endo H before analysis by PAGE.

METHODS. HeLa cells on 35-mm dishes (6×10^5 cells per dish) were infected with vTF-7 at a multiplicity of 10 plaque-forming units per cell. The virus was allowed to absorb for 30 min in serum-free DMEM at 37 °C with frequent rocking. Cells were then transfected with the indicated amounts of plasmid DNAs as described previously²⁹ using a modification of the lipofection procedure³⁰, employing dimethyldioctadecyl ammonium bromide as the cationic lipid (J.K.R., L.B. and M. Whitt, manuscript in preparation). Three hours after infection, 1.5 ml DMEM with 10% fetal bovine serum was added to the cells. Four hours after transfection, cells were metabolically labelled by incubation for 30 min in 0.5 ml methionine-free DMEM, containing 75 μ Ci [³⁵S]methionine. The cells were then lysed, or washed and incubated for 2 h in DMEM containing 2 mM unlabelled methionine. Proteins were immunoprecipitated²⁷ from cell lysates or medium using the monoclonal antibody OKT4. Endo H digestion was as described³¹ using 1 mU endo H in 0.15 M sodium citrate buffer (pH 5.5). Immunoprecipitated proteins were analysed by electrophoresis in SDS-10% polyacrylamide gels³².

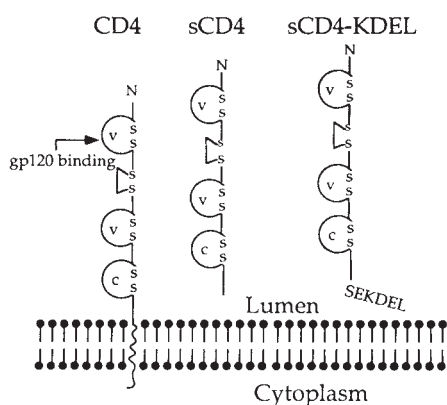


FIG. 1 Diagram of wild-type and soluble CD4 proteins. The domain structure of CD4 is illustrated along with the two mutant forms.

METHODS. Mutagenesis to generate cDNAs encoding the sCD4 and sCD4-KDEL mutants was performed as described²⁶, but employing single-stranded phagemid DNA. The CD4 constructs were generated using the plasmid pBS-CD4 (ref. 27). The plasmid pBS-sCD4 was generated using the oligonucleotide 5'-GTGCAGCCAAATGGCCTGAATCTGCTGGGGGCGTCGCC-3' to replace the codon for CD4 amino acid 374 with the stop codon TGA. The plasmid pBS-CD4-KDEL encoding sCD4-KDEL was generated by replacing the CD4 sequence encoding amino acids 374–381 with the sequence encoding SEKDEL and a stop codon using the oligonucleotide 5'-GTGCAGCCAAATGGCCAGCGAAAAGGACGAGCTCTAAGCCGGCCTCCTGCT-3'. Sequence changes in all mutated DNAs were confirmed by DNA sequencing²⁸.

cessed to endo H resistance after 2 h, even in the presence of excess sCD4-KDEL (Fig. 3c). This was the same amount of processing observed in the absence of sCD4-KDEL (Fig. 2b). Consistent with this observation, we found that sCD4-KDEL did not interfere with surface expression of wild-type CD4 (not shown). In addition to establishing that sCD4-KDEL did not interfere with CD4 transport, these results showed that the effect of sCD4-KDEL on gp120 transport was specific and not due to a general inhibition of exocytosis.

Because only a small fraction of gp120/41 is expressed at the cell surface, we could not quantify the effect of sCD4-KDEL on gp120/41 surface expression directly. We therefore used a more sensitive assay, the ability of surface gp120/41 to mediate

syncytia formation in CD4⁺ HeLa cells⁶⁻⁸. We observed that in the absence of sCD4-KDEL expression, most of the cells had fused to form large polykaryons (Fig. 4a). But when we expressed sCD4-KDEL with gp120/41 (Fig. 4b), no polykaryons were formed, presumably because the gp160 was never expressed at the cell surface. Control experiments showed that the amounts of gp160 synthesized with and without sCD4-KDEL were similar (not shown). Coexpression of sCD4 instead of sCD4-KDEL with gp160 gave a partial inhibition of syncytia formation (not shown). This effect presumably reflects partial saturation of CD4 binding sites on gp120 and is expected from earlier studies showing that soluble CD4 can block HIV infection¹⁸⁻²².

Baltimore has suggested a procedure of 'intracellular immunization', a form of gene therapy in which genes encoding proteins capable of interfering with HIV growth might be introduced into a patient's T cells. These proteins would then protect the T cells from subsequent HIV infection⁹. The use of a gene encoding sCD4-KDEL in such a procedure could have advantages over other procedures involving CD4 such as direct administration of sCD4 (refs 18-22). Because the sCD4-KDEL/gp160 complexes would remain intracellular and eventually be degraded, possible toxic side effects of circulating sCD4 would be avoided. In addition to blocking gp120/41 transport and release of circulating gp120, expression of excess sCD4-KDEL should inhibit assembly of infectious virus at the plasma membrane. Experiments designed to assess the effects of sCD4-KDEL expression on T-lymphocyte growth and on HIV replication are in progress.

The other possibilities suggested for intracellular immunization are HIV *rev*²³, *tat*²⁴ and *gag*²⁵ genes containing dominant negative mutations interfering with HIV replication. It might prove advantageous to use sCD4-KDEL instead of dominant

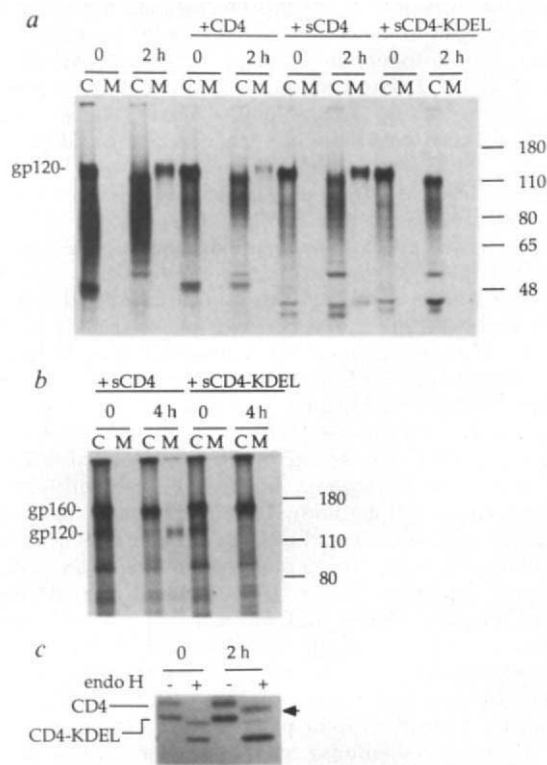


FIG. 3 Secretion of gp120 is blocked specifically by sCD4-KDEL. *a*, HeLa cells infected with vTF-7 were transfected with 1.5 μ g pBSsgp120 alone (encoding HIV-1 gp120), or in combination with 4.5 μ g pBSsCD4, pBSsCD4, or pBSsCD4-KDEL as indicated. Cells were pulse-labelled with [³⁵S]methionine for 30 min and immunoprecipitates prepared before or after a 2-h chase from cell lysates (C) or culture medium (M) using polyclonal sheep anti-gp120 serum (AIDS Research and Reference Program 288). Proteins were resolved by electrophoresis on a 10% SDS-polyacrylamide gel and visualized by autoradiography. Positions of protein markers are shown ($M_r \times 10^{-3}$). *b*, HeLa cells were infected with vTF-7 and transfected with 2.5 μ g pBSgp160 alone or with 5.0 μ g pBSsCD4 or pBSsCD4-KDEL DNAs as indicated. The experiment was performed as described in *a*, except that the chase period was 4 h to allow gp120 to accumulate in the medium. *c*, HeLa cells were infected with vTF-7 and transfected with 2.0 μ g pBSsCD4 and 6.0 μ g pBSsCD4-KDEL. The pulse-chase protocol and treatment of immunoprecipitates with endo H were as described in Fig. 2b. The arrow indicates the position of partially endo H-resistant CD4.

METHODS. The region encompassing the HIV-1 *env* gene (BH10 clone³³) was cloned into the *Eco*RI and *Sst*I sites of the plasmid pBSSK⁺ (Stratagene) under control of the T7 promoter using an *Eco*RI site introduced 99 nucleotides upstream from the *env* initiation codon (at an *Av* site) and an *Sst*I site following the gp41 encoding sequence. The plasmid was designated pBSgp160. A plasmid encoding only the soluble gp120 portion of gp160 was generated by mutagenesis of pBSgp160 with the oligonucleotide 5'-AGAAGAGTGGTGCAGTGAATTCAGAGCAGTGGGAA-3', to introduce a TGA codon in place of the AGA codon at amino acid 508. Pulse-chase labelling, immunoprecipitations and endo H digestions were as described for Fig. 2.

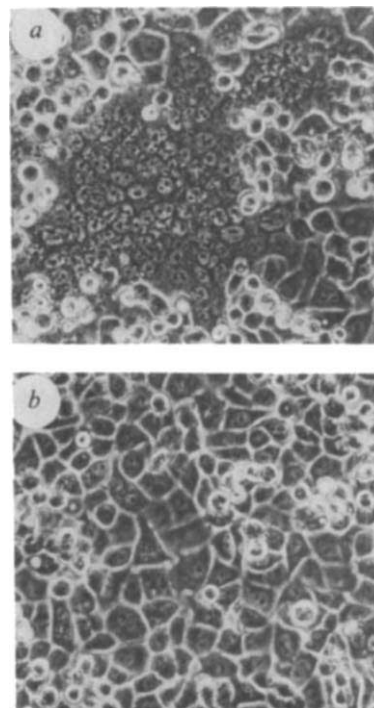


FIG. 4 Expression of sCD4-KDEL blocks the fusion activity of gp120/41. The photographs show extensively fused CD4⁺ HeLa cells expressing gp160 (*a*) or unfused cells expressing gp160 and sCD4-KDEL (*b*).

METHODS. CD4⁺ HeLa cells were infected with vTF-7 and transfected (as described in Fig. 2) with 5.0 μ g pBSgp160 alone or 5.0 μ g pBSgp160 and 10.0 μ g pBSsCD4-KDEL plasmid. The cells were incubated for 18 h at 37 °C and photographed under phase-contrast microscopy.

negative viral gene products because HIV should be unable to evade the block in glycoprotein transport without losing the ability to bind its receptor. Any specific viral mutations in gp120 overcoming the block in retention by sCD4-KDEL would probably also prevent subsequent infection of CD4⁺ cells. The procedure of blocking transport of a viral glycoprotein with a soluble, KDEL-containing form of the viral receptor might also be applicable to other virus-host systems. □

Received 13 March; accepted 4 May 1990.

1. Gallo, R. C. *et al. Science* **224**, 500–503 (1984).
2. Barre-Sinoussi, F. *et al. Science* **220**, 868–871 (1983).
3. Sattentau, Q. J. & Weiss, R. A. *Cell* **52**, 631–633 (1988).
4. Maddon, P. J. *et al. Cell* **42**, 93–104 (1985).
5. Maddon, P. J. *et al. Cell* **47**, 333–348 (1986).
6. Lifson, J. D., Reyes, G. R., McGrath, M. S., Stein, B. S. & Engelman, E. G. *Science* **232**, 1123–1127 (1986).
7. Lifson, J. D. *et al. Nature* **323**, 725–728 (1986).
8. Sodroski, J., Goh, W. C., Rosen, C., Campbell, K. & Haseltine, W. A. *Nature* **322**, 470–474 (1986).
9. Baltimore, D. *Nature* **335**, 395–396 (1988).
10. Hoxie, J. A. *et al. Science* **234**, 1123–1127 (1986).
11. Kawamura, I. *et al. J. Virol.* **63**, 3748–3754 (1989).
12. Willey, R. L., Bonifacino, J. S., Potts, B. J., Martin, M. A. & Klausner, R. D. *Proc. natn. Acad. Sci. U.S.A.* **85**, 9580–9584 (1988).
13. Munro, S. & Pelham, H. R. B. *Cell* **48**, 899–907 (1987).
14. Fuerst, T. R., Niles, E. G., Studier, F. W. & Moss, B. *Proc. natn. Acad. Sci. U.S.A.* **83**, 8122–8126 (1986).
15. Iarentino, A. L. & Maley, F. *J. biol. Chem.* **249**, 811–817 (1974).
16. Kornfeld, R. & Kornfeld, S. A. *Rev. Biochem.* **54**, 631–664 (1985).
17. Dunphy, W. G. & Rothman, J. E. *Cell* **42**, 13–21 (1985).
18. Smith, D. H. *et al. Science* **238**, 1704–1707 (1987).
19. Fisher, R. A. *et al. Nature* **331**, 76–78 (1988).
20. Hussey, R. E. *et al. Nature* **331**, 78–81 (1988).
21. Deen, K. C. *et al. Nature* **331**, 82–84 (1988).
22. Trautwein, A., Luke, W. & Karjalainen, K. *Nature* **331**, 84–86 (1988).
23. Malim, M. H., Bohrlin, S., Hauber, J. & Cullen, B. R. *Cell* **58**, 205–214 (1989).
24. Green, G., Ishino, M. & Loewenstein, P. M. *Cell* **58**, 215–223 (1989).
25. Trono, D., Feinberg, M. B. & Baltimore, D. *Cell* **59**, 113–120 (1989).
26. Kunkel, T. A. *Proc. natn. Acad. Sci. U.S.A.* **82**, 488–492 (1985).
27. Shaw, A. S. *et al. Cell* **59**, 627–636 (1989).
28. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).
29. Whitt, M., Chong, L. & Rose, J. K. *J. Virol.* **63**, 3569–3578 (1989).
30. Feigner, P. L. *et al. Proc. natn. Acad. Sci. U.S.A.* **84**, 7413–7417 (1987).
31. Rose, J. K. & Bergman, J. E. *Cell* **34**, 513–524 (1983).
32. Laemmli, U. K. *Nature* **227**, 680–684 (1970).
33. Ratner, L. *et al. Nature* **313**, 277–284 (1985).

ACKNOWLEDGEMENTS. We thank D. Brown, B. Crise, A. Shaw and M. Whitt for helpful comments on the manuscript. This work was supported by the NIH.

Fc receptor phosphorylation during receptor-mediated control of B-cell activation

Walter Hunziker, Terry Koch, J. Andrew Whitney & Ira Mellman*

Department of Cell Biology, Yale University School of Medicine, 333 Cedar Street, PO Box 3333, New Haven, Connecticut 06510, USA

It is well known that Fc receptors for IgG (FcRII) on macrophages mediate the endocytosis of antibody–antigen complexes and signal the release of inflammatory and cytotoxic agents¹. FcRII are also expressed at high levels on B cells where they are less involved in endocytosis than in modulating B-cell activation by membrane immunoglobulins^{2,3}. Although crosslinking of membrane immunoglobulins can result in B-cell differentiation and proliferation through stimulation of phospholipase C, mobilization of intracellular Ca²⁺, and activation of protein kinase C, crosslinking FcR with membrane immunoglobulins confers a dominant inhibitory signal that prevents or aborts activation^{2–6}. This form of regulation may have a role in the induction of tolerance by IgG⁷ and in controlling the B-cell repertoire by anti-idiotypes^{8,9}. The

different functions of FcR on B cells and macrophages may reflect the fact that these cell types express closely related but distinct FcR isoforms. We have recently found that the main lymphocyte FcR isoform, FcRII-B1, is unable to mediate endocytosis by way of coated pits and coated vesicles owing to an in-frame insertion of 47 amino acids in its cytoplasmic tail^{10,11}. Here we show that this insert, absent from the FcRII-B2 macrophage isoform, also contains serine phosphorylation sites that may have a role in the ability of FcR to regulate B-cell activation through membrane immunoglobulins.

Although FcR on both macrophages and B cells must be involved in the transduction of signals across the plasma membrane, little is known about how these signals are transmitted or whether specific FcRII isoforms are involved. Given the role of receptor phosphorylation in many types of signal transduction, and the roles of phosphorylation cascades in B-cell activation, we sought to determine whether one or more FcRII isoforms were phosphorylated. Complementary DNAs encoding FcRII-B1 and -B2 were transfected into two FcR⁺ cell lines, Madin–Darby canine kidney and Chinese hamster ovary cells^{10,11}. To determine whether either receptor could be phosphorylated, FcR were immunoprecipitated from P_i-labelled transfectants using a polyclonal anti-FcRII antiserum that recognizes all isoforms. FcRII-B1 (Fig. 1a, lanes 1 and 3) but not FcRII-B2 (lanes 2 and 4) was phosphorylated in both cell lines.

As FcRII-B1 is normally the only isoform expressed in B cells (neither FcRII-B2 nor a second macrophage-specific isoform FcRII-A is detected in splenic B cells or A20 lymphoma cells (ref. 12, 13 and T. K. *et al.*, manuscript in preparation)) we next determined whether endogenous FcR was also subject to phosphorylation. As shown in Fig. 1b, basally phosphorylated FcRII-B1 could be immunoprecipitated both from labelled A20 cells and from primary B lymphocytes (lanes 1–6), either using the polyclonal anti-FcRII antibody (lanes 1, 2, 5 and 6) or an anti-peptide antiserum specific for FcRII-B1 (lane 4). Labelled receptor was not precipitated using pre-immune sera or an FcRII-A specific anti-peptide antiserum (lanes 11 and 3). The degree of FcRII-B1 phosphorylation was increased by several times in both cell types by treatment with phorbol 12-myristate-13-acetate (PMA; lanes 2 and 6), an agent known to enhance B-cell activation by stimulating protein kinase C (refs 14, 15).

We also examined receptor phosphorylation in mouse peritoneal macrophages induced by thioglycollate; these do not express FcRII-B1 but do express FcRII-B2 and FcRII-A (ref. 13, and T. K. *et al.*, in preparation). Unexpectedly, phosphorylated FcR was detected in ³²P-labelled macrophages using the polyclonal antiserum. Use of the anti-peptide antisera specific for FcRII-B1 and FcRII-A showed that FcRII-A was labelled in the presence or absence of PMA (lanes 7–10). Together with the lack of FcRII-B2 phosphorylation in transfected cell lines, these results indicate that only FcRII-A is phosphorylated in macrophages.

The ability of PMA to increase FcRII-B1 phosphorylation in B cells suggested that phosphorylation may correlate with activation through crosslinking of membrane immunoglobulin (mIg). Crosslinking by mIg is known to induce breakdown of phosphatidylinositol 4,5-bisphosphate to inositol trisphosphate (IP₃) and 1,2-diacylglycerol (DAG) (refs 16–20), which then mediate Ca²⁺ mobilization and translocation of PKC to the plasma membrane^{21,22}. The triggering of these events is aborted when FcR are crosslinked with mIg using anti-immunoglobulin IgGs possessing intact Fc domains, as opposed to F(ab')₂ fragments of anti-immunoglobulin¹⁸.

We thus determined whether crosslinking mIg alone—or together with FcR—had any effect on the phosphorylation state of FcR on A20 cells²³, a lymphoma line thought to mediate signal transduction through mIg in a manner similar to B lymphocytes²⁴. To crosslink mIg without FcR, cells were incubated with F(ab')₂ fragments of IgG antibodies to mouse

* To whom correspondence should be addressed.

immunoglobulin in the presence of $^{32}\text{P}_i$. As shown in Fig. 2a, crosslinking of mIg alone markedly stimulated FcR phosphorylation (lane 3), similarly to PMA treatment (lane 1). On the other hand, little if any increase in phosphorylation was found if the cells were incubated with intact IgG antibodies to mouse immunoglobulin (α -immunoglobulin IgG, lane 4) which cross-link mIg together with FcR²⁵. An occasional slight stimulation of FcR phosphorylation observed after incubation with anti-immunoglobulin IgG could reflect the transient activation of a small fraction of PKC under this condition^{18,26,27}.

To confirm that the anti-immunoglobulin F(ab')₂ and anti-immunoglobulin IgG antibodies could signal a response in A20 cells, we determined whether crosslinking mIg alone—but not with surface FcRII-B1—resulted in the production of interleukin-2 (IL-2)²⁴. Culture supernatants from A20 cells grown in the presence of anti-immunoglobulin F(ab')₂ or anti-immunoglobulin IgG were tested for their ability to support growth of the IL-2-dependent helper T cell line HT-2. As shown in Fig. 2b, only supernatants from A20 cells cultured in anti-immunoglobulin F(ab')₂ allowed proliferation of HT-2 cells; these supernatants were as effective as those from a cell line (D10) that constitutively secretes IL-2. In contrast, supernatants from A20 grown in the presence of anti-immunoglobulin IgG or in the absence of any anti-immunoglobulin antibody were completely unable to support HT-2 proliferation. Taken

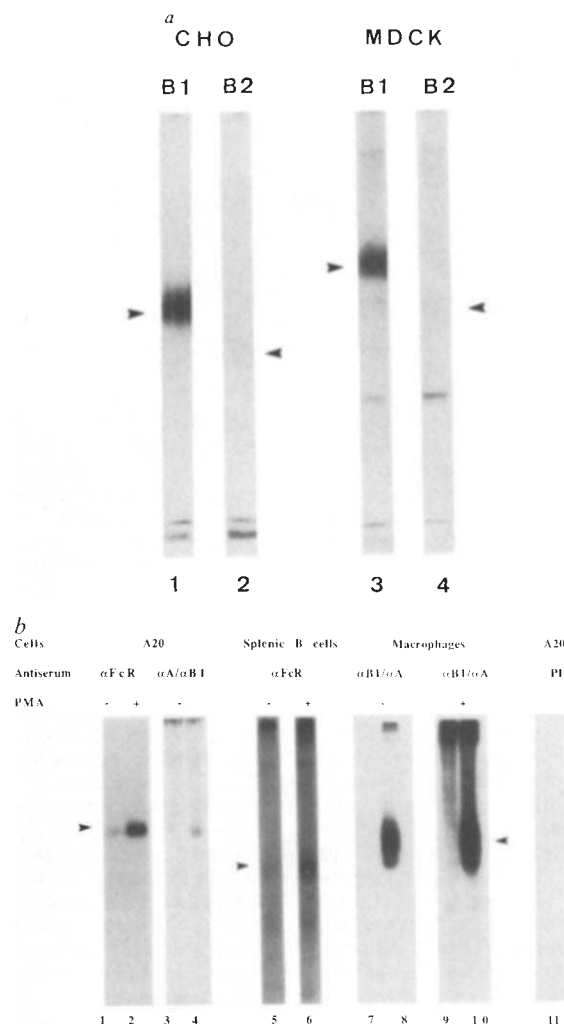
together, these results confirm a close relationship between the conditions used for stimulating FcRII-B1 phosphorylation and A20 cell activation through mIg crosslinking.

We next determined whether ligand binding to FcR itself induced receptor phosphorylation or prevented its ability to be phosphorylated by other means. As shown in Fig. 2c, incubation of A20 cells with saturating concentrations of IgG-immune complexes²⁸ did not stimulate FcR phosphorylation (lane 1). But, ligand binding *per se* did not inhibit phosphorylation by the separate crosslinking of mIg (lane 3). In the presence of IgG complexes phosphorylation occurred even if intact anti-immunoglobulin IgG antibody was used (lane 2), presumably because crosslinking of mIg to FcR was prevented by occupancy of the receptor's ligand-binding site by IgG complexes. PMA was also able to induce the phosphorylation of FcR bound to ligand (lane 4). Thus, separate occupancy of FcR and mIg does not prevent either B-cell activation^{2,6} or stimulation of receptor phosphorylation.

FcR phosphorylation represented a rapid response to mIg crosslinking: significant phosphorylation occurred within 5 min after addition of anti-immunoglobulin F(ab')₂ and was concentration dependent, reaching saturation at $\sim 5 \mu\text{g ml}^{-1}$ (Fig. 3b).

To characterize the possible involvement of PKC in mediating FcR phosphorylation, A20 cells were pretreated with

FIG. 1 a, Phosphorylation of FcRII-B1 in transfected Chinese hamster ovary (CHO) and Madin-Darby canine kidney (MDCK) cells. Immunoprecipitation of FcR from stably transfected CHO and MDCK cells expressing FcRII-B1 (lanes 1 and 3) or FcRII-B2 (lanes 2 and 4) after labelling with ^{32}P -orthophosphoric acid. The figure was assembled using lanes from different gel systems; arrows indicate the mobility of FcRII-B1 or -B2. Apparent molecular weights: FcRII-B1 $\sim 60\text{K}$; FcRII-B2: $\sim 50\text{K}$. b, Fc receptor phosphorylation in A20 cells, splenic B-cells and macrophages. Immunoprecipitation of FcR from A20 cells (lanes 1–4 and 11), mouse splenic B cells (lanes 5 and 6) and mouse peritoneal macrophages (lanes 7–10) labelled with ^{32}P -orthophosphoric acid in the presence (+) or absence (–) of PMA. Immunoprecipitations were done with anti-FcR antiserum (lanes 1, 2, 5 and 6), or anti-peptide antibodies specific for FcRII-B1 (αB1 , lanes 4, 7 and 9) or FcRII-A (αA , lanes 3, 8 and 10), or pre-immune serum (PI, lane 11). The figure was assembled using lanes from different gel systems; arrows indicate the mobility of FcRII-A or -B1, respectively. Apparent molecular weights: FcRII-B1 $\sim 55\text{K}$; FcRII-A $\sim 45\text{K}$. METHODS. a, Stably transfected cells (5×10^6) expressing FcRII-B1 or FcRII-B2^{10,11} were washed three times with labelling buffer (1.4 mM CaCl₂, 5.4 mM KCl, 0.8 mM Mg(SO₄)₂, 116.4 mM NaCl, 26.2 mM NaHCO₃, 55 mM D-glucose and 10 mM HEPES pH 7.0). After depleting phosphates by incubation in labelling buffer for 15 min at 37 °C, cells were transferred to 2 ml labelling buffer containing 1 mCi carrier-free ^{32}P -orthophosphoric acid (New England Nuclear) for 60 min at 37 °C. Lysis of cells and immunoprecipitation with anti-FcR antiserum was as described¹¹, except that the TX-114 phase-separation and pre-adsorption to fixed *Staphylococcus aureus* were omitted; in addition, all buffers were supplemented with 20 mM NaF, 100 μM sodium vanadate and 2 mM EDTA. Immunoprecipitates were analysed by SDS-PAGE (10% acrylamide) and autoradiography using intensification screens. Average exposure time was 12 h. Transfected cell lines expressed the following number of receptors per cell: FcRII-B1: $\sim 2 \times 10^5$ (MDCK) and $\sim 10^6$ (CHO); FcRII-B2: $\sim 10^5$ (MDCK) and $\sim 4 \times 10^5$ (CHO). b, Mouse peritoneal macrophages were obtained from thioglycollate-treated BALB/c mice³⁶. Mouse B cells were isolated from spleens of BALB/c mice and depleted of T cells and macrophages as described³⁶. Mouse peritoneal macrophages (5×10^6), splenic B cells (5×10^6) and A20 B-lymphoma cells (2.5×10^7) were washed and phosphate-depleted as described in the legend to Fig. 1. Phosphate labelling was carried out in 1 ml labelling buffer containing 0.5 mCi ^{32}P -orthophosphoric acid in the presence or absence of $1 \mu\text{g ml}^{-1}$ PMA (Sigma) for 60 min at 37 °C. Cells were then lysed and immunoprecipitated with a polyclonal anti-FcR antiserum that recognizes all three receptor isoforms (αFcR ; Fig. 1 legend), or with anti-peptide antibodies specific for the unique cytoplasmic tail sequences of FcRII-B1 (αB1) or FcRII-A (αA). Anti-peptide antibodies were produced in rabbits by bilateral injection into the popliteal lymph node using peptides coupled to keyhole limpet haemocyanin. The anti-FcRII-A antiserum was obtained using a synthetic peptide encoding its complete cytoplasmic domain (26 residues, RRNLQTPREYWRKSLIRKHQAPQDK (single-letter code)). The anti-FcRII-B1 antiserum was generated using a peptide derived from the 47-amino-acid



insertion in the FcRII-B1 cytosolic domain³⁰ (PEEVGEYRQPSGLSAC). Details concerning the production and specificity of the anti-peptide antisera will be published elsewhere (T.K. *et al.*, in preparation). Immunoprecipitates were analysed by SDS-PAGE and autoradiography using intensification screens. Average exposure time was 6 h.

staurosporine, a potent inhibitor of PKC²⁹. Staurosporine (80 nM) strongly inhibited PMA- and anti-immunoglobulin F(ab')₂-induced phosphorylation (Fig. 3c, lanes 3 and 4). Also, phosphorylation was no longer induced by either PMA or anti-immunoglobulin F(ab')₂ after depleting cellular PKC by growth of A20 cells in PMA (200 nM) for 48 h (not shown). Direct PKC activation by PMA could, however, overcome the inhibition of phosphorylation in cells that had FcR crosslinked to mIg (Fig. 3d, lane 3). These results suggest a direct or indirect involvement of PKC in mediating FcR phosphorylation on B cells.

To determine which residues were phosphorylated, phosphoamino acid analysis was carried out on ³²P-labelled FcR isolated from PMA- or anti-immunoglobulin F(ab')₂-stimulated A20 cells, and PMA-induced mouse splenic B cells or peritoneal macrophages. As shown in Fig. 4, serine was the residue mainly phosphorylated in FcRII-B1 isolated from A20 cells stimulated by PMA (Fig. 4a) or anti-immunoglobulin F(ab')₂ (Fig. 4b). Trace amounts of phosphothreonine were present in FcRII-B1 isolated from PMA-stimulated splenic B cells (Fig. 4c) or in FcRII-A from PMA-stimulated peritoneal macrophages (Fig. 4d). In FcRII-B1, the modified serine residue(s) are likely to be located in the 47-amino-acid insertion³⁰, the only domain

not present in the otherwise identical FcRII-B2 isoform. Of the seven serines found in this insertion, residues Ser 239 (Arg-Gln-Pro-Ser) and/or a cluster of three serine residues at positions 257-259 (Pro-Thr-Ser-Ser-Ser-Pro) are likely targets for phosphorylation. The cytoplasmic tail of FcRII-A carries only two closely linked serine residues which are flanked by basic amino acids (Arg-Lys-Ser-Leu-Ser-Ile-Arg-Lys); phosphorylation must occur on one or both residues.

It is well established that B-cell activation results in the phosphorylation of a variety of proteins as a consequence of PKC activation^{17,21,31}. Although there are several suggestions that FcR interacts with elements in the mIg signalling pathway, the molecular nature of the inhibitory FcR-mediated signal on B-cell activation is unknown. FcR phosphorylation could be involved in controlling coupling of mIg to a guanine nucleotide regulatory protein²⁷, or modulate FcR interactions with adenylate cyclase³², phospholipase A₂ (ref. 33) or a protein kinase as has been suggested for a macrophage cell line³⁴. The known regulatory function of FcR together with our finding that FcRII-B1 is phosphorylated in response to mIg cross-linking suggests a role for receptor phosphorylation in modulating B-cell activation. Only expression of mutant receptors lacking the phos-

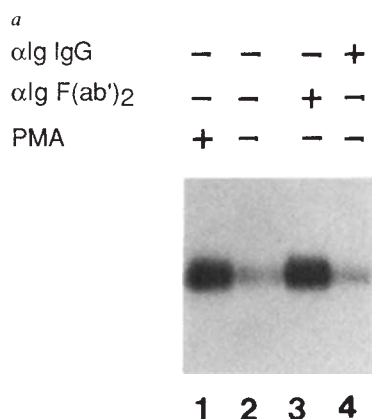
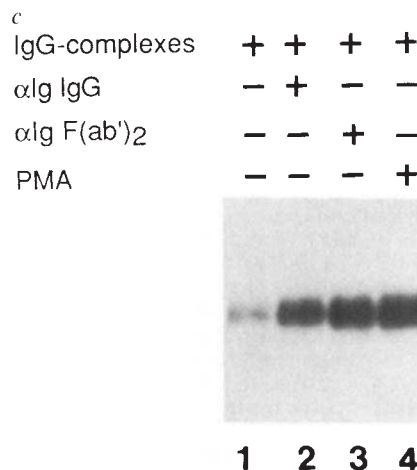
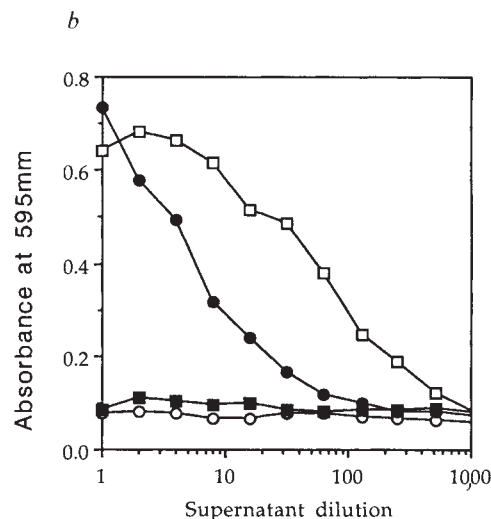


FIG. 2 Crosslinking membrane immunoglobulin results in FcRII-B1 phosphorylation in B cells. a, Effect of crosslinking mIg alone or to FcR on FcR phosphorylation. Immunoprecipitation of FcR from A20 cells labelled with ³²P-orthophosphoric acid alone (lane 2) or in the presence of PMA (lane 1), anti-immunoglobulin Fab₂ fragments (αIg F(ab')₂, lane 3) or anti-immunoglobulin IgG (αIg IgG; lane 4). b, Interleukin-2 production by A20 cells in response to crosslinking mIg. Proliferation of IL-2-dependent HT-2 cells after culturing in serial dilutions of culture supernatants from A20 cells in the presence of anti-immunoglobulin F(ab')₂ (●), anti-immunoglobulin IgG (■), or in the absence of any anti-immunoglobulin antibodies (○). Supernatants from the constitutively IL-2 secreting cell line D10 were used as a positive control (□). Proliferation of HT-2 cells was determined by a colorimetric assay measuring the conversion of MTT to formazan²⁴. c, Preincubation of A20 cells with IgG complexes. Immunoprecipitation of FcR from A20 cells preincubated with IgG complexes followed by labelling with ³²P-orthophosphoric acid alone (lane 1) or supplemented with anti-immunoglobulin Ig (lane 2), anti-immunoglobulin F(ab')₂ (lane 3) or PMA (lane 4).

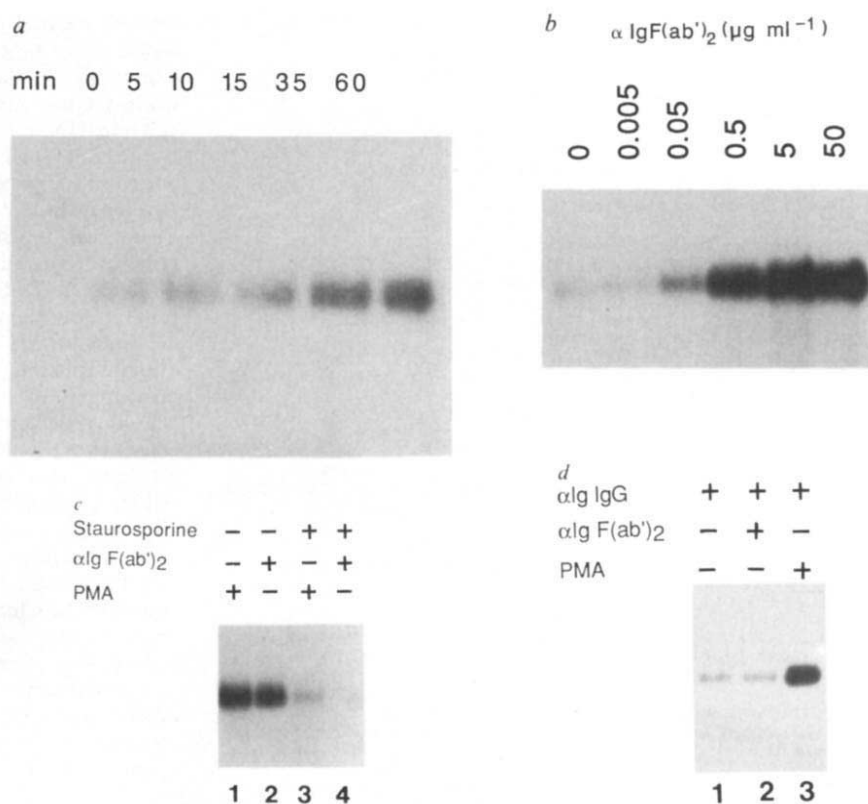
METHODS. a, A20 cells (2.5×10^7) were washed, phosphate-depleted as described in Fig. 1 legend and then incubated in 1 ml labelling buffer alone (-) or supplemented with $1 \mu\text{g ml}^{-1}$ PMA; $50 \mu\text{g ml}^{-1}$ F(ab')₂ fragments of goat anti-mouse immunoglobulin or $100 \mu\text{g ml}^{-1}$ rabbit anti-mouse immunoglobulin IgG for 30 min at 37 °C. Both anti-mouse immunoglobulin reagents were from Jackson ImmunoResearch and were dialysed against 10 mM HEPES pH 7.0, 150 mM NaCl. ³²P-orthophosphoric acid (0.5 mCi) was then added and the incubation continued for 60 min, after which cells were lysed and FcR immunoprecipitated as described above. b, Culture supernatants were obtained from A20 cells (2.5×10^7) incubated for 20 h in 1 ml αMEM supplemented with 5% FCS and $50 \mu\text{M}$ 2-mercaptoethanol in the presence of $50 \mu\text{g ml}^{-1}$ F(ab')₂ fragments of goat anti-mouse immunoglobulin, $100 \mu\text{g ml}^{-1}$ rabbit anti-mouse immunoglobulin IgG or in the absence of any anti-immunoglobulin antibodies. Lymphokine activity in



serial 1:1 dilutions of the supernatants was assayed using the IL-2-dependent HT-2 cell line and the colorimetric MTT (Sigma) assay, essentially as previously described²⁴. c, washed and phosphate-depleted A20 cells (2.5×10^7) were preincubated in 1 ml labelling buffer containing $20 \mu\text{g ml}^{-1}$ rabbit anti-DNP:DNP-BSA IgG complexes²⁸ for 30 min at 37 °C. ³²P-orthophosphoric acid (0.5 mCi) alone (-) or supplemented with anti-immunoglobulin IgG ($100 \mu\text{g ml}^{-1}$), anti-immunoglobulin F(ab')₂ ($50 \mu\text{g ml}^{-1}$) or PMA ($1 \mu\text{g ml}^{-1}$) was then added and incubation was continued for 60 min before immunoprecipitation of FcR.

FIG. 3 Regulation of FcRII-B1 phosphorylation. *a*, Kinetics of receptor phosphorylation by anti-immunoglobulin F(ab')₂. Immunoprecipitation of FcR from A20 cells after pre-labelling with ³²P-orthophosphoric acid followed by incubation in the presence of anti-immunoglobulin F(ab')₂ for 0–60 min. *b*, Dependence of FcR phosphorylation on anti-immunoglobulin F(ab')₂ concentration. Immunoprecipitation of FcR from A20 cells after pre-labelling with ³²P-orthophosphoric acid followed by incubation with 0–50 µg ml⁻¹ anti-immunoglobulin F(ab')₂ for 60 min. *c*, Inhibition of anti-immunoglobulin F(ab')₂ and PMA induced FcR phosphorylation by staurosporine. Immunoprecipitation of FcR from A20 cells pre-treated without (lanes 1 and 2) or with (lanes 3 and 4) staurosporine and then labelled with ³²P-orthophosphoric acid in the presence of PMA (lanes 1 and 3) or anti-Ig F(ab')₂ (lanes 2 and 4). *d*, Preincubation with anti-immunoglobulin IgG. Immunoprecipitation of FcR from A20 cells after pre-treating with anti-immunoglobulin IgG and then adding ³²P-orthophosphoric acid alone (lane 1) or supplemented with anti-immunoglobulin F(ab')₂ (lane 2) or PMA (lane 3).

METHODS. *a*, A20 cells were washed in labelling buffer as described and then labelled with 0.5 mCi/ml ³²P-orthophosphoric acid for 60 min. Anti-immunoglobulin F(ab')₂ (50 µg ml⁻¹) was then added and after 0–60 min 1 ml aliquots (2.5 × 10⁷ cells) were removed, cells pelleted and immediately frozen in liquid nitrogen. FcR were immunoprecipitated as described above. *b*, A20 cells (2.5 × 10⁷) were labelled with ³²P-orthophosphoric acid for 60 min as described in *c*. Anti-immunoglobulin F(ab')₂ was then added to give final concentrations of 0–50 µg ml⁻¹. After 30 min at 37°C, cells were processed for immunoprecipitation. *c*, Washed and phosphate-depleted A20 cells (2.5 × 10⁷) were preincubated in 1 ml labelling buffer alone or supplemented with 80 nM staurosporine (Calbiochem) for 30 min at 37°C. ³²P-orthophosphoric acid (0.5 mCi) containing anti-immunoglobulin F(ab')₂ (50 µg ml⁻¹) or PMA (1 µg ml⁻¹) was then added



and the incubation continued for an additional 60 min, after which FcR were immunoprecipitated. *d*, Washed and phosphate-depleted A20 cells (2.5 × 10⁷) were preincubated in 1 ml labelling buffer containing 100 µg ml⁻¹ anti-immunoglobulin IgG for 30 min at 37°C. ³²P-orthophosphoric acid (0.5 mCi) alone (–) or supplemented with anti-immunoglobulin F(ab')₂ (50 µg ml⁻¹) or PMA (1 µg ml⁻¹) was then added and the incubation continued for 60 min before FcR immunoprecipitation.

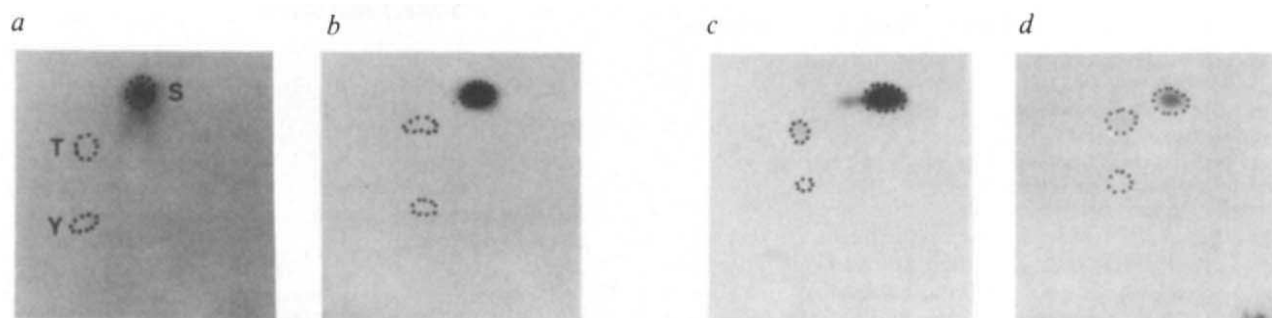


FIG. 4 Phosphoamino acid analysis of phosphorylated Fc receptors. Thin layer electrophoresis of phosphoamino acids obtained from FcR isolated from cells labelled with ³²P-orthophosphoric acid. *a*, A20 cells stimulated with PMA; *b*, A20 cells treated with anti-immunoglobulin F(ab')₂; *c*, mouse splenic B cells treated with PMA; and *d*, mouse macrophages treated with PMA. The positions of phosphoserine (S); phosphothreonine (T) and phosphotyrosine (Y) standards are circled and representatively labelled in panel *a*.

phorylation target sites in FcR⁺ B cells will, however, conclusively show whether phosphorylation is a cause or an effect of receptor-mediated modulation of B-cell activation.

The finding that the macrophage FcRII-A isoform is also phosphorylated indicates the possible involvement of this isoform in signal transduction on macrophages. Murine natural killer cells, which share many aspects of the signal transduction pathway with macrophages but lack the ability to internalize complexed IgG, predominantly express the FcRII-A isoform³⁵.

METHODS. A20 cells, peritoneal macrophages and splenic B cells were labelled in the presence of PMA (1 µg ml⁻¹) or anti-immunoglobulin F(ab')₂ (50 µg ml⁻¹) and immunoprecipitated as described in Fig. 1*b*. FcR was purified by gel electrophoresis, eluted and phosphoamino acids analysed by two-dimensional thin layer electrophoresis³⁷.

It is conceivable therefore, that on macrophages different receptor isoforms mediate endocytosis (FcRII-B2)^{10,11} and signal transduction (FcRII-A). □

Received 11 October 1989; accepted 27 March 1990.

1. Mellman, I. *Curr. Opin. Immunol.* **1**, 16–25 (1988).
2. Klaus, G. G. B., Bijsterbosch, M. K., O'Garra, A., Harnett, M. M. & Rigley, K. P. *Immunol. Rev.* **99**, 19–38 (1987).
3. Cambier, J. C. *et al. Immunol. Rev.* **95**, 37–57 (1987).

4. Berridge, M. J. *Rev. Biochem.* **56**, 159-193 (1987).
5. Phillips, N. E. & Parker, D. C. *J. Immun.* **132**, 627-632 (1984).
6. Sinclair, N. R. S. & Panoskaltis, A. *Immunol. Today* **8**, 76-79 (1987).
7. Waldschmidt, T. J., Borel, Y. & Vitetta, E. S. *J. Immun.* **131**, 2204-2209 (1983).
8. Sinclair, N. R. S. *J. exp. Med.* **129**, 1183-1192 (1969).
9. Kohler, H., Richardson, B. C., Rowley, D. A. & Smyk, S. *J. Immun.* **119**, 1979-1984 (1977).
10. Miettinen, H. M., Rose, J. K. & Mellman, I. *Cell* **58**, 317-327 (1989).
11. Hunziker, W. & Mellman, I. *J. Cell Biol.* **109**, 3291-3302 (1989).
12. Lewis, V. A., Koch, T., Plutner, H. & Mellman, I. *Nature* **324**, 372-375 (1986).
13. Ravetch, J. V. *et al. Science* **234**, 718-725 (1986).
14. Monroe, J. G. & Kass, M. J. *J. Immun.* **135**, 1674 (1985).
15. Klaus, G. G. B., O'Garra, A., Bijsterbosch, M. K. & Holman, M. *Eur. J. Immun.* **16**, 92 (1986).
16. Coggeshall, K. M. & Cambier, J. C. *J. Immun.* **133**, 3382-3386 (1984).
17. Coggeshall, K. M. & Cambier, J. C. *J. Immun.* **134**, 101-107 (1985).
18. Bijsterbosch, M. K. & Klaus, G. G. B. *J. exp. Med.* **162**, 1825-1836 (1985).
19. Bijsterbosch, M. K., Meade, C. J., Turner, G. A. & Klaus, G. G. B. *Cell* **41**, 999-1006 (1985).
20. Ransom, J. T., Harris, L. K. & Cambier, J. C. *J. Immun.* **137**, 708-715 (1986).
21. Nel, A. E. *et al. Biochem. J.* **233**, 145-149 (1986).
22. Chen, Z. Z., Coggeshall, K. M. & Cambier, J. C. *J. Immun.* **136**, 2300-2304 (1986).
23. Kim, K. J., Kanellopoulos-Langevin, C., Merwin, R. M., Sachs, D. H. & Asofsky, R. *J. Immun.* **122**, 549-554 (1979).
24. Justement, L. B., Kreiger, J. & Cambier, J. C. *J. Immun.* **143**, 881-889 (1989).
25. Parker, D. C. *Immunol. Rev.* **52**, 115-130 (1980).
26. Wilson, H. A. *et al. J. Immun.* **138**, 1712-1718 (1987).
27. Ringley, K. P., Harnett, M. M. & Klaus, G. G. B. *Eur. J. Immunol.* **19**, 481-485 (1989).
28. Ukonen, P., Lewis, V., Marsh, M., Helenius, A. & Mellman, I. *J. exp. Med.* **163**, 952-971 (1986).
29. Tamaoki, T. *et al. Biochem. biophys. Res. Commun.* **135**, 397-402 (1986).
30. Hogarth, P. M. *et al. Immunogenetics* **26**, 161-168 (1987).
31. Hornbeck, P. & Paul, W. E. *J. biol. Chem.* **261**, 14817-14824 (1986).
32. Ryan, J. L. & Henkart, P. A. in *Regulatory Mechanisms in Lymphocyte Activation* (ed. Lucas, D. O.) 4444 (Academic, New York, 1977).
33. Suzuki, T. R. *et al. Biochemistry* **19**, 6037-6044 (1980).
34. Hirata, Y. & Suzuki, T. *Biochemistry* **26**, 8189-8195 (1987).
35. Perussia, B. *et al. J. exp. Med.* **170**, 73-86 (1989).
36. Pure, E., Witmer, M. D., Lum, J. B., Mellman, I. & Unkeless, J. C. *J. Immun.* **139**, 4152-4158 (1987).
37. Stern, D. F., Heffernon, P. A. & Weinberg, R. A. *Molec. cell. Biol.* **6**, 1729-1740 (1986).

ACKNOWLEDGEMENTS. We thank David Stern and Andrey Shaw for help and advice with phosphoamino acid analysis, James Drake for help with the HT-2 assay and for reading the manuscript, and Heini Miettinen for CHO cell lines expressing FcR isoforms. We thank Ari Helenius, Janet Larkin, Benjamin Podbilewicz and Yvette Rheault for discussions, and Ann Curly-Whitehouse for photographic work. These studies were supported by a fellowship from the Damon Runyon-Walter Winchell Cancer Research Fund (to W.H.) and by grants from the NIH and the International Immunology Research Institute (to I.M.).

T-cell clones from a type-1 diabetes patient respond to insulin secretory granule proteins

Bart O. Roep*, Susan D. Arden†, René R. P. de Vries* & John C. Hutton†

* Department of Immunohaematology and Blood Bank, University Hospital, Leiden, The Netherlands

† Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Cambridge, UK

T LYMPHOCYTES reactive to pancreatic β -cells are thought to have a central role in the autoimmune process leading to type 1 (insulin-dependent) diabetes¹⁻⁶, but the molecular targets of these T cells have not yet been defined. As identification of such antigens may enable measures to be developed to prevent the disease, we have characterized an antigen that is recognized by insulinoma membrane-reactive T-cell clones established from a newly diagnosed type-1 diabetes patient⁷. Subcellular fractionation studies using rat insulinoma indicate that the antigenic determinant recognized by one of these clones is an integral membrane component of the insulin secretory granule. After a 5,000-fold purification, we have defined the antigen as a monomer of relative molecular mass 38,000. As granular membrane proteins are transiently exposed on the cell surface during exocytosis, their accessibility to components of the immune system may be a function of the secretory activity of β -cells.

Homogenates of transplantable rat insulinoma tissue⁸ were centrifuged on a discontinuous Nycodenz density gradient. The following fractions were collected: cytosolic constituents, plasma membrane, insulin secretory granules and a pellet composed of a mixture of lysosomes, mitochondria and endoplasmic reticulum (as defined by analysis of marker proteins) (Table 1). Each fraction was tested for its ability to stimulate the prolifera-

tion of a panel of T-cell clones isolated from the peripheral blood of a newly diagnosed type-1 diabetic⁷. These CD4-expressing, HLA-DR1-restricted T-cell clones were generated using a crude membrane preparation from the rat insulinoma cell line RINm5F as antigen. They also recognize highly purified canine islet cells and a human fetal pancreas preparation enriched for β -cells, implying that the antigen is a conserved β -cell constituent rather than tumour-specific or species-specific. There was a marked proliferative response to the fraction enriched in insulin secretory granules with two clones, 1C6 and 1C10 (Table 1). Some reactivity was observed with the pelleted proteins, but the extent of proliferation was commensurate with contamination by insulin secretory granules. The secretory granule fraction was also subjected to Percoll density-gradient centrifugation⁹ which gave a twofold increase in the specific activity of the granule marker and a 2-5-fold decrease in specific activities of the other marker proteins. The proliferative response of clones 1C6 and 1C10 to these purified granules was not reduced, indicating that the T-cell responses were not the result of contamination of the granule fraction by a minor cellular component (data not shown). The secretory granule preparation was further fractionated into granule matrix and membrane components. Clones 1C6 and 1C10 responded only to the membrane fraction, and continued to respond after extraction procedures that remove extrinsic membrane proteins (Table 2).

The molecular weight of the antigen recognized by 1C6 was determined using SDS-PAGE of granule membranes followed by T-cell proliferation assays on electroeluted proteins. The response was directed against fractions with a relative molecular

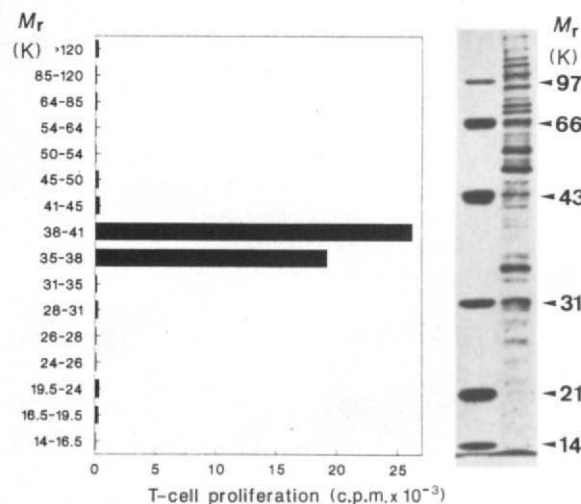


FIG. 1 Determination of the molecular weight of the granule membrane protein recognized by T-cell clone 1C6. The proliferative response to eluted protein ($1 \mu\text{g ml}^{-1}$) is shown alongside a silver-stained reference polyacrylamide of the granule membrane fraction ($5 \mu\text{g}$ protein). Relative molecular masses (M_r) were derived from the indicated standards (in thousands (K)). Granule membranes were prepared from insulinoma granules by lysis in $10 \text{ mM NH}_4\text{HCO}_3$ containing 1 M KCl (see Table 2 legend). The washed membrane pellet ($200 \mu\text{g}$ protein) was resuspended in $50 \mu\text{l}$ 125 mM Tris-HCl containing 0.2% sodium dodecyl sulphate (SDS) and 0.001% bromophenol blue, heated for 10 min at 60°C and loaded onto a 1-cm wide lane on a $15 \times 15 \times 0.3 \text{ cm}$ polyacrylamide gel (12.5% (w/v) acrylamide, 0.1% N,N' -methylenebisacrylamide²⁷). After electrophoresis the gel was cut into 0.5-cm or 1-cm slices and each slice was electroeluted at 120 V for 60 min into 50 mM Tris , 50 mM glycine , 1 M NaCl , 0.1% SDS ($75 \mu\text{l}$) using an LKB Extraphor apparatus (Pharmacia). The electroeluted proteins were precipitated using 3 volumes of acetone and stored at -70°C overnight before centrifugation at $11,000g$ for 5 min . Each pellet was washed twice in acetone, air-dried and resuspended by sonication in $10 \text{ mM NH}_4\text{HCO}_3$ ($100 \mu\text{l}$). Protein concentrations were determined (legend to Table 1) and samples diluted to $1 \mu\text{g ml}^{-1}$ in culture medium for the T-cell proliferation assay. The overall recovery of the electroeluted proteins was 70% and each eluted fraction contained $5\text{--}20 \mu\text{g}$ protein. The extent of purification of the antigen at this stage relative to initial insulinoma protein was $5,000\text{-fold}$.

TABLE 1 Subcellular distribution of T-cell antigens

Fraction	Enzyme activity (nmol min ⁻¹ mg ⁻¹)				T-cell proliferation (c.p.m.)		
	Alkaline phosphatase	Aryl sulphatase	Cytochrome oxidase	NADPH-cytochrome c reductase	Carboxypeptidase H	1C6	1C10
Cytosol	2.8	17.1	0.0	0.5	6.7	145 ± 71	3,630 ± 2,723
Plasma membranes	720.7	22.1	28.8	26.3	9.0	372 ± 138	6,422 ± 1,284
Secretory granules	91.5	27.4	135.7	27.8	24.2	23,737 ± 2,848	24,537 ± 2,944
Pellet	53.3	110.3	460.0	94.0	16.1	16,513 ± 4,128	15,883 ± 635

The T-cell clones 1C6 and 1C10 showed a strong proliferative response to insulinoma subcellular fractions enriched for the secretory granule marker, carboxypeptidase H. The response to fractions enriched for cytosolic proteins, plasma membrane constituents (marker, alkaline phosphatase) were appreciably lower. The T-cell response to the pellet fraction which contained endoplasmic reticulum (NADPH-cytochrome c reductase), lysosomes (aryl sulphatase) and mitochondria (cytochrome oxidase) results from contamination by secretory granule components. Subcellular fractionation: All steps were at 4 °C unless specified. Transplantable rat insulinoma tissue (3–5g) was homogenized in 270 mM sucrose, 10 mM MES-KOH, 1 mM EGTA, 1 mM MgSO₄ solution, pH 6.5 (20 ml), centrifuged for 6 min at 1,770g to remove cell debris and nuclei. The supernatant (20 ml) was layered onto a three-stepped (5 ml per step) density gradient made by mixing 19.2% (w/v) Nycodenz, 170 mM sucrose, 10 mM MES-KOH solution with the homogenization medium in ratios 1:3, 1:1 and 1:0. The gradient was centrifuged for 60 min at 100,000g in a Beckman SW28 rotor and material recovered from the supernatant (cytosol), the 0/4.8% Nycodenz interface (light membranes), the 9.6/19.2% interface (insulin secretory granules) and the pellet. The particulate fractions were washed twice (by resuspension in homogenization medium and centrifugation at 100,000g for 25 min) and stored at 1–2 mg protein ml⁻¹ in 270 mM sucrose 10 mM MES, pH 6.5, at –70 °C. Marker proteins were determined as before^{8,10}. T-cell proliferation was assayed as previously described⁷. Briefly, 10⁴ T cells and 5 × 10⁴ irradiated HLA-DR1, 1 mononuclear cells (antigen-presenting cells) were cultered in 96-well flat-bottom microtitre plates in triplicate in the presence of 5 µg ml⁻¹ antigen for three days. [³H]thymidine was added and cells were incubated for 16 h, then collected on a glass fibre filter. Liquid scintillation counting was used to measure the c.p.m.; Values are indicated as c.p.m. ± s.e.m. Protein was determined with Pierce BCA reagent after solubilizing membrane samples by heating for 5 min at 60 °C in 20 µl 0.2% sodium dodecyl sulphate in 10 mM Tris-HCl, pH 8. Results are from a single experiment and are representative of data obtained on three separate occasions.

mass (*M_r*) of 38,000 (38K) under non-reducing conditions (Fig. 1). Reduction of disulphide bonds in the sample before electrophoresis did not affect the apparent size of the antigen.

The cell-type distribution of the antigen recognized by 1C6 is shown in Table 3. Strong positive reactions were seen with pituitary, adrenals, and a neuroblastoma cell line, as well as with insulin-producing tissue. These tissues, together with the pancreatic islets, are members of the diffuse neuroendocrine system and have a number of morphological and molecular features in common^{10–13}. One of these is the storage of bioactive polypeptides in intracellular storage vesicles and their secretion by exocytosis. The reactive tissues differ in this respect from most of the non-reactive tissues in the series. It seems unlikely, however, that the antigen is a functional component of the exocytotic process, because tissue from the exocrine pancreas did not induce a proliferative response.

Our experiments have identified an islet-cell antigen that is recognized by cloned T cells—as opposed to circulating autoantibodies—from a type-1 diabetes patient. Islet-cell auto-

antibodies are often present in the peripheral blood of type-1 diabetes patients years before the onset of the disease^{14,15} and considerable effort has been directed towards their characterization. These studies have shown that there are several antigenic targets distributed in different subcellular compartments of the pancreatic islet cells. Some of the antigens seem to be β -cell specific, others are also found in other endocrine tissues and elsewhere^{12,13}. However, they have not been identified at the molecular level and at present insulin is the only diabetes-related autoantigen that has been defined¹⁶. Other putative antigens include a 64K membrane-associated protein precipitated by diabetic sera¹⁷, and a series of gangliosides¹⁸. It is unlikely that such islet-cell autoantibodies are involved in the pathogenesis of type-1 diabetes as the titres of such autoantibodies in patients after treatment with cyclosporin A do not correlate with β -cell function³. Also, in type-1 diabetics receiving pancreatic transplants from an identical twin or sibling, insulinitis recurs in the absence of circulating islet-cell autoantibodies⁴.

There is increasing evidence that T lymphocytes present in the inflamed pancreatic islets are important in the pathogenesis of the disease^{4–7}. Infiltrated T cells are only seen at the time of clinical manifestation of the disease if identifiable β -cells are

TABLE 2 Membrane localization of the antigen recognized by 1C6 and 1C10

Antigen	Extraction	T-cell proliferation*	
		1C6	1C10
Homogenate	—	43,090 ± 1,293	7,517 ± 977
Medium	—	187 ± 91	390 ± 351
Pellet 0	Low salt	33,053 ± 2,327	15,587 ± 6,235
Supernatant 0	Low salt	2,875 ± 690	303 ± 151
Pellet 1	High salt	12,592 ± 4,064	9,092 ± 2,819
Supernatant 1	High salt	468 ± 334	60 ± 12
Pellet 2	Alkaline	5,017 ± 1,054	1,380 ± 138
Supernatant 2	Alkaline	90 ± 17	122 ± 31

* Values are c.p.m. ± s.e.m.

Insulinoma secretory granules (1–2 mg protein) prepared as in Table 1 were extracted sequentially by sonication (for 20 s; MSE sonicator) in the following ice-cold solutions: 10 mM NH₄HCO₃ (low salt), 10 mM NH₄HCO₃ containing 2 mM EDTA and 1 M NaCl (high salt) or 0.5 M Na₂CO₃ (alkaline) (5 ml). After each extraction the material was centrifuged at 240,000g for 30 min, and the pellet washed by resuspension in 5 ml 10 mM NH₄HCO₃ and centrifugation as before. Pellets were resuspended at each step in 10 mM NH₄HCO₃ (50 µl) and assayed for protein and antigenic activity (as described in the legend to Table 1). Results are from a single experiment and are representative of data obtained on three separate occasions.

TABLE 3 Tissue distribution of the antigen recognized by 1C6

Source of antigen	T-cell proliferation*
Insulinoma	31,277 ± 4,379
Neuroblastoma	31,323 ± 1,879
Pituitary	10,482 ± 3,669
Adrenals	23,473 ± 1,643
Thyroid	877 ± 430
Gastric fundus	728 ± 473
Medium	1,182 ± 142

* Values are c.p.m. ± s.e.m.

Solid rat tissue was pulverized in liquid nitrogen, then sonicated in ice-cold 10 mM Tris-HCl, pH 7.4. Cellular debris was removed by centrifugation at 1,700g for 10 min and a membrane fraction prepared by centrifugation at 100,000g for 60 min. The membranes were resuspended in culture medium (5 µg ml⁻¹) and tested for antigenicity in a T-cell proliferation assay as described. A negative response (judged as radioactivity incorporation of less than threefold of that observed with T cells and antigen-presenting cells in the absence of antigen) was obtained with rat fibroblasts, liver, lung, heart muscle, skeletal muscle and a human exocrine pancreatic tumour.

still present⁴. Experimental models indicate that both CD4⁺ and CD8⁺ T cells from diabetic animals can be toxic to β -cells or transfer the disease to healthy recipients¹⁹⁻²².

There is a correlation between functional activity of the pancreatic β -cell and development of type-1 diabetes^{23,24}. The phenomenon of glucose toxicity and the amelioration of clinical symptoms following functional repression might be linked to changes in cell-surface expression of insulin granule membrane components and their consequent interaction with elements of the immune system. In this context, it is interesting that the antigen defined here localizes to the secretory granule membrane.

Although this study does not define the role of T cells in the immunopathology of type-1 diabetes, it is possible that CD4⁺ T cells, like the clones described in this study, are pathogenic. For example, CD4⁺ T cells that react with myelin basic protein are mediators in experimental allergic encephalomyelitis, an autoimmune disease of the central nervous system²⁵. Although the precise mechanism of autoimmune attack in this illness is unknown, the identification of such clones and their target epitopes has led to novel therapeutic approaches for treatment or prevention of the disease^{25,26}. Such measures may ultimately provide a means of preventing type-1 diabetes in susceptible individuals. □

Received 18 January; accepted 4 April 1990.

1. Bottazzo, G. F. *et al.* *New Engl. J. Med.* **313**, 353-360 (1985).
2. Stiller, C. R. *et al.* *Science* **223**, 1362-1367 (1984).
3. Mandrup-Poulsen, T. *et al.* *Lancet* **i**, 599-602 (1989).
4. Sibley, R. K., Sutherland, D. E. R., Goetz, F. & Michael, A. F. *Lab. Invest.* **53**, 132-144 (1985).
5. De Berardinis, P. *et al.* *Lancet* **ii**, 823-824 (1988).
6. Haskins, K., Portas, M., Bergman, B., Lafferty, K. & Bradley, B. *Proc. natn. Acad. Sci. U.S.A.* **86**, 8000-8004 (1989).
7. Van Vliet, E., Roep, B. O., Meulenbroek, L., Bruining, G. J. & De Vries, R. R. P. *Eur. J. Immun.* **19**, 213-216 (1989).
8. Guest, P. C., Pipeleers, D., Rossier, J., Rhodes, C. J. & Hutton, J. C. *Biochem. J.* **264**, 503-508 (1989).
9. Hutton, J. C., Penn, E. J. & Peshavaria, M. *Diabetologia* **23**, 365-373 (1982).
10. Hutton, J. C. *Diabetologia* **32**, 271-281 (1989).
11. Day, I. N. M. *Molec. cell. Probes* **1**, 275-295 (1987).
12. Sugiura, M. *et al.* *Diab. Res.* **3**, 111-114 (1986).
13. Sugiura, M. *et al.* *Diab. Res.* **4**, 63-66 (1987).
14. Bottazzo, G. F., Florin-Christensen, A. & Doniach, D. *Lancet* **ii**, 1279-1283 (1974).

15. Bruining, G. J., Molenaar, J. L. & Grobbee, D. E. *Lancet* **i**, 1100-1103 (1989).
16. Palmer, J. P. *et al.* *Science* **222**, 1337-1339 (1983).
17. Baekkeskov, S. *et al.* *Nature* **298**, 167-169 (1982).
18. Powers, P. C., Rabizadeh, A., Akeson, R. & Eisenbarth, G. S. *Endocrinology* **114**, 1338-1343 (1984).
19. Prud'homme, G. J., Fuks, A., Colle, E. & Guttman, R. D. *Diabetes* **33**, 801-803 (1984).
20. Miller, B. J., Appel, M. C., O'Neill, J. J. & Wicker, L. S. *J. Immun.* **140**, 52-58 (1988).
21. Charlton, B. & Mandel, T. E. *Diabetes* **37**, 1108-1112 (1988).
22. Greiner, D. L. *et al.* *J. exp. Med.* **166**, 461-475 (1987).
23. Dampé, O., Andersson, A., Björk, E., Hallberg, A. & Karlsson, F. A. *Diabetes* **38**, 1326-1328 (1989).
24. Gotfredson, C. F., Buschard, K. & Frandsen, E. K. *Diabetologia* **28**, 933-935 (1985).
25. Acha-Orbea, H. *et al.* *Cell* **54**, 263-273 (1988).
26. Janeway, C. A. *Nature* **341**, 482-483 (1989).
27. Laemmli, U. K. *Nature* **227**, 680-685 (1970).

ACKNOWLEDGEMENTS. The authors thank Drs Elaine Bailyes, Wouter Hazenbos and Jan Bruining for their contribution and the British Diabetic Association for supporting this project.

The LDL receptor pathway delivers arachidonic acid for eicosanoid formation in cells stimulated by platelet-derived growth factor

Andreas J. R. Habenicht, Peter Salbach, Matthias Goerig, Wolfgang Zeh, Uwe Janssen-Timmen, Christine Blattner, Weiling C. King* & John A. Glomset*

University of Heidelberg, 69-Heidelberg, Bergheimerstrasse, 58, FRG

*Howard Hughes Medical Institute Laboratory, SL-15,

University of Washington, Seattle, Washington 98195, USA

ANIMAL cells can convert 20-carbon polyunsaturated fatty acids into prostaglandins (PGs) and leukotrienes. These locally produced mediators of inflammatory and immunological reactions act in an autocrine or paracrine fashion^{1,2}. Arachidonic acid (AA), the precursor of most PGs and leukotrienes, is present in the form of lipid esters within plasma lipoproteins and cannot be synthesised *de novo* by animal cells. Therefore, AA or its plant-derived precursor, linoleic acid, must be provided to cells if PGs or leukotrienes are to be formed². Because several classes of lipoproteins, including low-density lipoproteins (LDL), very-low-density lipoproteins, and chylomicron remnants, are taken up by means of the LDL receptor³⁻⁵, and because LDL and very-low-density lipoproteins⁶⁻⁸, but not high-density lipoproteins⁶, stimulate PG synthesis, we have suggested previously that PG formation is directly linked to the LDL pathway⁶. Using fibroblasts with the receptor-negative phenotype of familial hypercholesterolaemia and anti-LDL receptor antibodies, we show here that LDL deliver AA for PG production and that an LDL receptor-dependent feedback mechanism inhibits the activity of PGH synthase, the rate-limiting enzyme of PG synthesis. These results indicate that the LDL pathway has a regulatory role in PG synthesis, in addition to its well-known role in the maintenance of cellular cholesterol homeostasis.

To test whether the AA in LDL lipid esters is used as a substrate for the production of PGs, we reconstituted native LDL with [¹⁴C]AA-labelled cholesteryl esters (CE)⁹. This generated LDL particles (recLDL-[¹⁴C]AA-CE) with a defined CE fatty acid composition. Addition of recLDL-[¹⁴C]AA-CE (8-80 µg protein per ml) to quiescent fibroblasts did not lead to significant production of [¹⁴C]6-keto-PGF_{1α} (the stable hydrolysis product of prostacyclin¹⁰) or [¹⁴C]PGE₂ (Fig. 1a). By contrast, cells stimulated with platelet-derived growth factor (PDGF) formed significant amounts of both labelled prostacyclin and PGE₂ (Fig. 1b). The extent of incorporation of the LDL-derived [¹⁴C]AA into PGs reached a plateau at ≤8 µg LDL protein per ml, indicating that the LDLs were taken up by a high-affinity mechanism. Further experiments provided direct evidence that the classical LDL pathway of Brown and Goldstein³ delivers AA for PG production. Fibroblasts from three unrelated patients with the receptor-negative phenotype of homozygous familial hypercholesterolaemia (FH) were unable to produce significant amounts of PGs when recLDL-[¹⁴C]AA-CE was added (Fig. 1a, b). But the mutant cells did form PGs when non-esterified [¹⁴C]AA was added, indicating that their failure to form PGs from LDL was a consequence of the receptor defect, rather than of a deficiency in the enzymes of PG synthesis¹¹. Moreover, normal fibroblasts incubated with an antibody directed against the LDL receptor also failed to produce PGs in response to LDL (Fig. 2a), and chloroquine, a

TABLE 1 LDL and AA inhibit V_{max} of PGH synthase in PDGF-stimulated Swiss 3T3 fibroblasts

	V_{max} (fmol PGE ₂ per µg protein per min)	K_m (µM AA)
Control	24 ± 9	9.4 ± 1.9
PDGF	387 ± 87	8.2 ± 2.0
PDGF + LDL	70 ± 21	7.4 ± 0.9
PDGF + AA	8 ± 3	6.4 ± 1.7

Cells were cultured, microsomes prepared, and kinetic parameters of PGH synthase determined as previously described¹¹. Cells were stimulated with PDGF for 12 h and then either incubated in the presence of 40 µg ml⁻¹ LDL protein or 10 µM AA for 6 h. Data represent means of quadruplicate samples ± s.d.

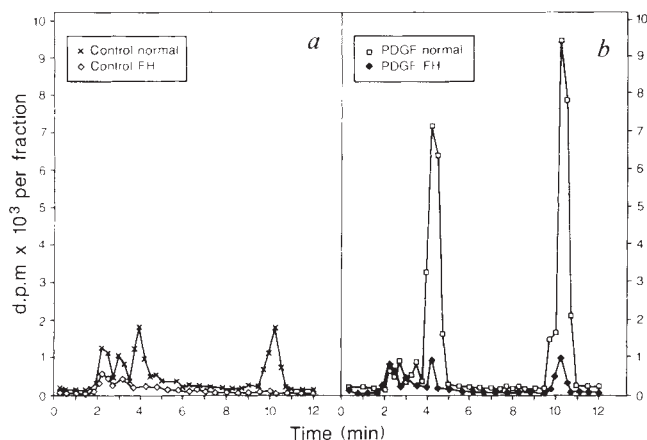


FIG. 1 Formation of PGs from recLDL-[^{14}C]AA-CE in normal and FH fibroblasts. FH cells with the receptor-negative phenotype³ (GM 02000 E, NIGMS human genetic mutant cell repository, Coriell Institute for Medical Research, New Jersey, USA) or normal fibroblasts were used. Normal and FH cells were stimulated with PDGF or normal fibroblasts were not (a).

METHODS. Cells (6×10^5 cells per 60-mm dish) were cultured as described and stimulated with 6 ng ml^{-1} of PDGF⁶. After 17 h recLDL-[^{14}C]AA-CE at $10 \mu\text{g}$ LDL protein per ml (specific activity, 2.12×10^5 d.p.m. per μg protein) was added for 6 h. RecLDL-[^{14}C]AA-CE was prepared as described⁹ using cholesteryl-[1- ^{14}C]arachidonate (specific activity, $54.9 \text{ mCi mmol}^{-1}$; NEN). Radioactivity in the PGs was determined after extraction¹¹ and high-pressure liquid chromatography on μ -Bondapak- C_{18} columns (Waters) in 67.2% $17 \text{ mM H}_3\text{PO}_4/32.8\%$ acetonitrile, pH 3.5 (1.7 ml per min). Retention times ranged from 2.5 to 4.7 min for 6-keto-PGF $_{1\alpha}$ and from 8.7 to 10.8 min for PGE $_2$. Chromatograms represent samples from one normal individual or from GM 02000 E FH fibroblasts ($10 \mu\text{g}$ per ml recLDL-[^{14}C]AA-CE protein per 10^6 cells every 6 h).

lysosomal enzyme inhibitor, greatly inhibited the ability of normal fibroblasts to form prostacyclin and PGE $_2$ from LDL⁶ (Fig. 2b), but not from exogenous arachidonate (data not shown). These results demonstrate that LDLs stimulate PG production by a mechanism that depends on LDL receptor-mediated delivery of AA to the PG synthesis pathway.

The data of Figs 1 and 2 raised the intriguing possibility that the LDL pathway has a dual role: an immediate PG-stimulatory effect, dependent on the delivery of substrate AA to the PGH synthase, and a delayed inhibitory effect resulting from the self-inactivation of PGH synthase, which occurs in purified enzyme preparations¹². To test whether LDL inhibits the activity of PGH synthase, we preincubated 3T3 fibroblasts with native LDL to stimulate them to form PGs. Then, to assess the residual activity of PGH synthase, we changed the medium and incubated them in the presence of unesterified AA. During the preincubation, LDL stimulated the formation of PGE $_2$ in a concentration-dependent and time-dependent way (Fig. 3a, c). During the subsequent incubation with unesterified AA, the cells also formed PGE $_2$, but in quantities that were inversely proportional to those formed in response to LDL (Fig. 3b, d). Further experiments showed that the inhibitory effect of LDL could be blocked by chloroquine and that acetylated LDL that does not bind to the LDL receptor⁶ did not inhibit PGH synthase (data not shown). In control experiments to compare the effects of unesterified AA with those of LDL, we found both quantitative and kinetic differences. Unesterified AA was faster and more effective than LDL, both at stimulating PG production (Fig. 3c) and in causing an apparent inhibition of PGH synthase (Fig. 3d). Subsequent measurements of the kinetic parameters of PGH synthase in microsomes provided direct evidence that both LDL and unesterified AA inhibit this enzyme. The V_{max} for the enzyme was high in microsomes from PDGF-stimulated cells, as previously reported¹¹. But it was 80% lower in microsomes from cells that had been preincubated with LDL, with no change in the apparent Michaelis constant, K_m , for the enzyme

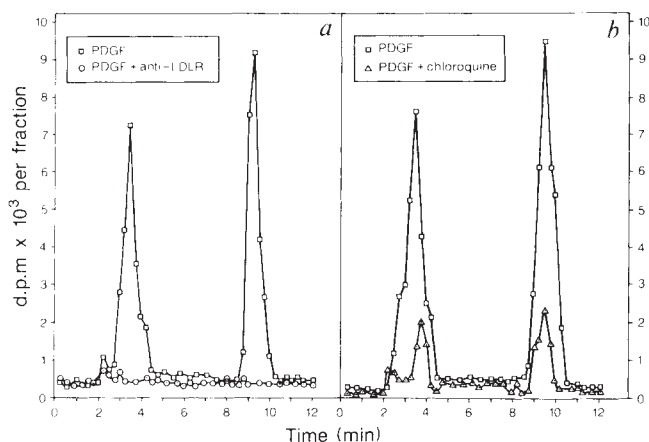


FIG. 2 Anti-LDL receptor antibody and chloroquine block PG synthesis from recLDL-[^{14}C]AA-CE in normal fibroblasts.

METHODS. This was a similar experiment to that of Fig. 1. Thirty minutes before addition of $8 \mu\text{g}$ recLDL-[^{14}C]AA-CE protein per ml, $200 \mu\text{g}$ per ml anti-LDL receptor antibody or $200 \mu\text{g}$ per ml preimmune rabbit IgG were added (a). Thirty minutes before addition of $40 \mu\text{g}$ per ml recLDL-[^{14}C]AA-CE protein, $60 \mu\text{M}$ chloroquine was added⁶ (b). Chromatograms represent PGs formed from $8 \mu\text{g}$ per ml (a) or $40 \mu\text{g}$ per ml (b) recLDL-[^{14}C]AA-CE per 10^6 cells every 6 h. A 20-fold excess of LDL receptor antibody with respect to LDL had been shown to block by 90% the binding of [^{125}I]LDL to fibroblasts in a standard binding assay. LDLR, LDL receptor.

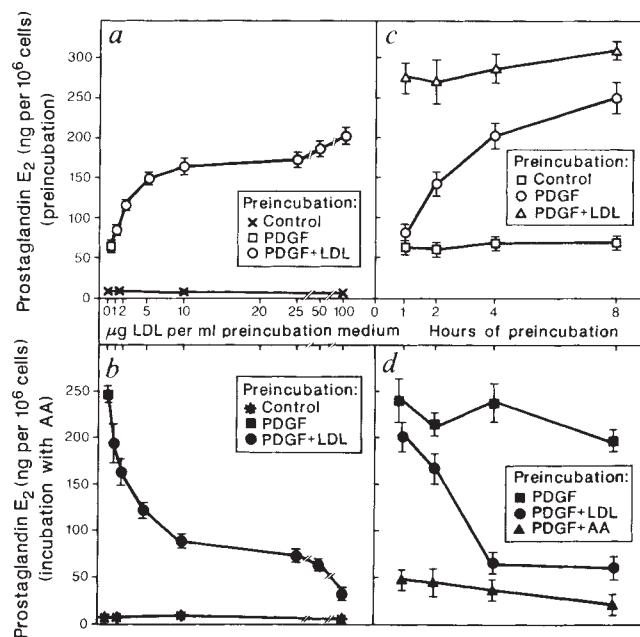


FIG. 3 LDL-mediated PGH synthase inhibition. Data represent means of three cultures $\pm$ s.d.

METHODS. 3T3 fibroblasts at a concentration 1.25×10^5 per 35-mm dish were cultured and stimulated as described. After 17 h, cells were incubated for 4 h with increasing concentrations of LDL (a), or for increasing time periods with $10 \mu\text{g}$ native LDL protein per ml or $10 \mu\text{M}$ AA¹¹ (c) and medium was removed for determination of PGE $_2$ ¹¹ (a and c, open symbols). Cultures were then washed to remove residual LDL or AA, incubated for 60 min in fresh medium containing $10 \mu\text{M}$ AA, and PGE $_2$ was determined as a measure of residual PGH synthase activity¹¹ (b, d, closed symbols).

(Table 1). The inhibitory effect of LDL and AA was reversible within 5–6 h of removal of the inhibitors (data not shown). Taken together, our results support three main conclusions: LDL stimulate PG production in PDGF-stimulated fibroblasts by providing substrate AA for the PGH synthase reaction; LDL-derived AA has a pronounced negative feedback effect on

PGH synthase; and both effects depend on the LDL receptor pathway³. Therefore, our results provide evidence of a new pathway for providing AA for PG production and a new regulatory role for the LDL receptor.

One of the important questions raised by this study is: which cells form prostacyclin and PGE₂ *in vivo* in an LDL-dependent way? Likely candidates would be cells that concomitantly express high numbers of the LDL receptor and the PGH and prostacyclin synthases, and other enzymes of the AA cascade¹¹. These cells would include replicating fibroblasts in wounds, as well as smooth muscle cells and monocytes/macrophages^{11,13,14} in atherosclerotic lesions^{6,15}. Consequently, replicating smooth muscle cells in proliferative atherosclerotic lesions of patients

afflicted with FH would lack the ability to form prostacyclin and PGE₂ in response to LDL. This raises the question of the possible biological role of the LDL-derived prostacyclin and PGE₂. Prostacyclin is the most potent anti-aggregatory molecule known¹⁰ whereas PGE₂ is a strong activator of adenylyl cyclase in fibroblasts^{16,17} and also a potent inhibitor of lymphocyte and macrophage activation^{2,11}. These biological activities, and the fact that the formation of prostacyclin and PGE₂ depends on the LDL receptor, could have important implications for the pathogenesis of atherosclerosis, as proliferating smooth muscle cells, activated lymphocytes, and differentiating macrophages are the principal cellular constituents of atherosclerotic plaques in patients afflicted with cardiovascular disease^{15,18-21}. □

Received 15 January; accepted 22 February 1990.

- Samuelsson, B., Dahlén, D. A., Lindgren, C. A., Rouzer, C. A. & Serhan, C. N. *Science* **237**, 1171-1176 (1987).
- Needleman, P., Turk, J., Jakschik, B. A. & Morrison, A. R. *Rev. Biochem.* **55**, 69-102 (1986).
- Brown, M. S. & Goldstein, J. L. *Science* **232**, 34-47 (1986).
- Mahley, R. *Science* **240**, 623-627 (1988).
- Koo, C. *et al. J. clin. Invest.* **81**, 1332-1340 (1988).
- Habernicht, A. J. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 1344-1348 (1986).
- Pomerantz, K. B., Tall, A. R., Feinmark, S. J. & Cannon, P. J. *Circulation Res.* **54**, 554-565 (1984).
- Spector, A. A. *et al. J. Lipid Res.* **26**, 288-297 (1985).
- Krieger, M. *Meth. Enzym.* **128**, 608-613 (1986).
- Moncada, S., Gryglewski, R., Bunting, R. & Vane, J. R. *Nature* **263**, 663-666 (1976).
- Goerig, M. *et al. J. Biol. Chem.* **263**, 19384-19391 (1988).

- Smith, W. L. & Lands, W. E. M. *Biochemistry* **11**, 3276-3283 (1972).
- Habernicht, A. J. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 921-924 (1989).
- Knight, B. L. & Soutar, A. K. *Eur. J. Biochem.* **125**, 407-413 (1982).
- Ross, R. *New Engl. J. Med.* **314**, 488-500 (1986).
- Rozenfurt, E. *et al. Cell* **34**, 265-272 (1983).
- Rozenfurt, E. *Science* **234**, 161-166 (1986).
- Heldin, C.-H. & Westermark, B. in *Growth Factors, Differentiation Factors and Cytokines* (ed. Habernicht, A.) 267-268 (Springer, Heidelberg, New York, Tokyo, 1989).
- Seifert, R. A., Schwartz, S. M. & Bowen-Pope, D. F. *Nature* **311**, 669-671 (1984).
- Rubin, K. *et al. Lancet* **i**, 1353-1356 (1988).
- Nowak, J. & FitzGerald, G. A. *Adv. Prost. Thrombox. Leukotr. Res.* **17**, 208-211 (1990).

ACKNOWLEDGEMENTS. We thank Dr Elaine Raines for the PDGF, Dr Thomas Innerarity for the anti-LDL receptor antibody, and Dr Joseph Goldstein for comments on the manuscript.

Sequence analysis and acute pathogenicity of molecularly cloned SIV_{SMM-PBj14}

Stephen Dewhurst*, Janet E. Embretson*, Daniel C. Anderson†, James I. Mullins*‡ & Patricia N. Fultz†||

* Harvard University, School of Public Health, Boston, Massachusetts 02115, USA

† Yerkes Regional Primate Research Center and Department of Pathology, Emory University, Atlanta, Georgia 30322, USA

‡ Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305-5402, USA

THE PBj14 isolate of simian immunodeficiency virus from sooty mangabey monkeys (SIV_{SMM-PBj14}) is the most acutely pathogenic primate lentivirus so far described, always causing fatal disease in pig-tailed macaques (*Macaca nemestrina*) within 8 days of inoculation¹. As a first step in identifying viral genes and gene products that influence pathogenicity, the SIV_{SMM-PBj14} genome was amplified by the polymerase chain reaction as 5' and 3' genomic halves of 5.1 and 5.8 kilobases, respectively, and molecularly cloned. DNA sequence analysis revealed a high degree of conservation with other SIVs, except for a 22-base-pair duplication in the enhancer region of the viral long terminal repeat which included a second binding site for the transcription factor NF- κ B. Of six genomic halves examined, four contributed to the formation of infectious virus that induced acute disease and death in pig-tailed macaques as early as 6 days post-inoculation, with pathology, disease syndromes and kinetics indistinguishable from those induced by the uncloned isolate. To our knowledge this is the first example of acute immunodeficiency disease induced by a molecularly defined lentivirus. Furthermore, the molecularly cloned SIV_{SMM-PBj14} viruses share with the uncloned virus cytopathicity for mangabey CD4⁺ cells, a property that may correlate with their observed pathogenicity *in vivo*.

SIV_{SMM} isolate PBj14 (SIV_{SMM-PBj14}) has resisted previous molecular cloning by conventional phage λ technology, presumably as a result of instability in prokaryotic host-vector systems. Therefore, we used the polymerase chain reaction (PCR) to increase the level of viral DNA, thereby permitting direct 'sub-cloning' into plasmid vectors. We cloned SIV_{SMM-PBj} genomes as two fragments, using as starting material cellular DNA from macaque peripheral blood mononuclear cells (PBMC) infected 7 days earlier with a biologically cloned virus (designated SIV_{SMM-PBj-bcl3}) which had been shown to be acutely pathogenic *in vivo*¹. Oligonucleotide primer pairs were selected from conserved regions of the genomes of SIV_{MAC} (rhesus macaque, *Macaca mulatta*), SIV_{SMM} (sooty mangabey monkey, *Cercocebus atys*) and SIV_{AGM} (African green monkey, *Cercopithecus aethiops*) (refs 2, 3; M. Murphey-Corb *et al.*, manuscript in preparation) to amplify regions spanning 5,086 base pairs (bp) from within the U3 region of the 5' long terminal repeat (LTR) into the *pol* gene and an overlapping fragment spanning 5,759 bp from the *pol* gene into the U5 region of the 3' LTR.

Using these procedures, we obtained intact clones of the 5' and 3' halves of the genome, and three clones from each genomic half were selected for further analysis. A conserved, unique *Bcl*I restriction site (corresponding to position 5,125 of SIV_{SMM-H-4}; ref. 2) was used to regenerate proviruses encompassing the entire virus transcription unit plus upstream positive regulatory sequences in the 5' LTR. Three infectious SIV_{SMM-PBj} chimaeras were generated (SIV_{SMM-PBj-1.9}, SIV_{SMM-PBj-4.9} and SIV_{SMM-PBj-4.41}); each replicated to high titres in PBMC from humans, pig-tailed macaques and mangabey monkeys, and induced cytopathic effects, including extensive cell death, in CEM $\times$ 174 cells (Table 1).

The complete DNA sequences of five of the six half-genomic fragments derived from SIV_{SMM-PBj} (5' clones 1 and 4; 3' clones 5, 9 and 41) were determined. The full-length infectious chimaeric virus construct, SIV_{SMM-PBj-4.41} (transcriptional unit length, 9,678 bp; total sequence, 9,995 bp), was adopted as a prototype for comparative purposes. Nucleotide divergence between clone 4.41 and the other clones consisted of point mutations only and ranged from 0.16% and 0.26% for 3' clones 5 and 9 to 0.43% for 5' clone 1. As expected, these percentages indicate a lower rate of divergence than previously observed for non-biologically cloned SIV_{MAC} isolates^{4,5}, but are 20-fold

|| To whom correspondence should be addressed.

TABLE 1 Biological properties of molecular clones of SIV_{SMM-PBJ14}

Clone 5' 3'	RT*	CEM x174	Cell-free infectivity†		Mang. PBMC	Inoculum‡ (TCID ₅₀)	Pathogenicity <i>in vivo</i>	
			Hum. PBMC	Mac. PBMC			Animal code	Day of death
1 5	—					Pig-tailed macaques		
2 9	—							
1 9	+	+	+	+	+		T323, F124	8, 55
4 9	+	+	+	+	+	1.5 × 10 ⁴	T325, 7D	49, —
4 41	+	+	+	+	+	1.5 × 10 ⁵	90E, F165	6, 6
						Rhesus macaques		
4 41						1.5 × 10 ⁵	RTP, RMP	—, —

* Plus sign, detection of cell-free reverse transcriptase activity and syncytia formation 5–20 days after transfection of CEMx174 cells.

† Plus sign, detection of supernatant reverse transcriptase activity 4–10 days after addition of 0.2–0.5 ml filtered virus-containing transfection supernatant to either 5 × 10⁶ CEMx174 cells or 1.4 × 10⁷ human, pig-tailed macaque or sooty mangabey monkey PBMC that had been stimulated for 3 days with phytohaemagglutinin P (Difco). After overnight adsorption of virus in 2.5 ml RPMI 1640 medium with supplements, the cells were washed and resuspended in 10 ml same medium.

‡ To generate virus stocks, supernatant fluids (collected 10–12 days after transfection of CEMx174 cells with cloned SIV_{SMM} DNA) were used to infect PBMC from normal human donors. Cell-free supernatants from the human PBMC cultures were collected on day 22, passed through 0.22 µm filters, and cryopreserved. At the same time the pig-tailed macaques were inoculated, the virus stocks were titrated in triplicate in 12-well plates by serial 10-fold dilutions and infection of human PBMC. Although the virus stocks had comparable reverse transcriptase activity (1.0 × 10⁶–1.3 × 10⁶ c.p.m. ml⁻¹), the TCID₅₀ (50% tissue culture infectious dose) obtained in human PBMC varied as much as 10-fold. The fact that the molecular clone with the lowest titre (SIV_{SMM-PBJ-4.9}) did not induce either inoculated animal to rapidly die probably was not related to the inoculum dose as uncloned SIV_{SMM-PBJ} induces acute disease and death uniformly over a range of virus titre from 5 × 10⁰ to 5 × 10³ TCID₅₀ (P.N.F., D.C.A., H. M. McClure, unpublished results).

|| Animals were housed in individual cages in an isolation facility and were monitored daily for signs of disease and overall activity and appearance. Macaque 7D is alive 7 months, and rhesus macaques RTP and RMP are well 5 months after inoculation.

higher than would be expected because of *Taq* polymerase errors alone (under similar conditions for amplification, we observed a *Taq* error rate of approximately 0.025% or a total of two base changes in more than 8 kilobases (kb) of plasmid DNA sequenced; P. Edmonson and C. deNoronha, personal communication). Thus, even a biologically cloned virus population contains some genetic diversity⁶.

The genomic organization of clone 4.41 was similar to that of other SIVs, and the deduced amino-acid sequences from nine open reading frames in the genome of SIV_{SMM-PBJ-4.41} revealed strongest similarities to that of SIV_{SMM-H-4} (Fig. 1b). Predictably,

regions encoding Gag and Pol proteins were most highly conserved, and the central region genes, *vif*, *vpx*, *rev*, *tat* and *vpr*, were present and well conserved. Interestingly, the SIV_{SMM-PBJ} 3' clone 5 lacks an initiator codon for *rev* owing to a point mutation, which most probably accounts for the replication-defectiveness of SIV_{SMM-PBJ-1.5}. Key structural features in *env* were also well conserved between SIV_{SMM-PBJ-4.41} and SIV_{SMM-H-4}, including cysteines (27 of 28), possible N-glycosylation sites (23 of 26), and the CD4-binding domain⁷ in the external Env protein and the fusion domain⁸ at the 5' end of the transmembrane protein. None of the SIV_{SMM-PBJ} sequences

FIG. 1 a, Schematic representation of the open reading frames and coding sequences in the SIV_{SMM-PBJ-4.41} genome. Vertical bars denote positions of stop codons. The position of point mutational changes that distinguish clones 1, 5 and 9 from clones 4 and 41 are indicated by vertical lines in the upper part of the figure. b, Amino-acid identity between SIV_{SMM-PBJ-4.41} and other primate lentiviruses. Sequences were obtained from the human retrovirus and AIDS database at the Los Alamos National Laboratory. Alignments were performed on a SUN microcomputer using FASTDB program¹⁹ (Intelligenetics) with default parameters. Dash, no comparison was performed as HIV-1 contains no *vpx* gene and SIV_{AGM} contains no *vpr* gene.

METHODS. PCR conditions were as recommended by Perkin-Elmer Cetus (PEC), except that the concentration of MgCl₂ was 2 mM and that of primers was 150 nM. Thirty-five cycles of amplification were performed in a PEC DNA Thermal Cycler using PEC Amplitaq *Taq* DNA polymerase. Each cycle used primer annealing steps at 55 °C for 2 min and extensions at 72 °C for 4 min. Primers consisted of a 3-bp 'clamp' (GAG) at the 5' end, followed by one or two restriction sites useful for later molecular cloning, followed by an SIV-specific sequence of 20–28 nucleotides. The sequences, restriction sites, and genome nucleotide (nt) positions relative to the SIV_{MAC-BK28} genome (M. Murphey-Corb *et al.*, manuscript in preparation) are:

5'-end primers

*Xma*I *Hind*III

SD-39 5'-GAG-CCCGGG-AAGCTT-CTGGCAGGAAGTTGTACCC-3' (nt 245–266)

*Sph*I *Nhe*I

SD-46 5'-GAG-GCATGC-GCTAGC-TAGCTTCTCTGGTACTAOC-3' (nt 5,300–5,276)

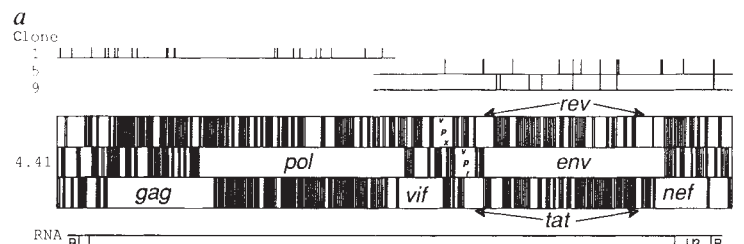
3'-end primers

*Sph*I *Hind*III

SD-29 5'-GAG-GCATGC-AAGCTT-GTACCTGCACATAAAGGAATAG-3' (nt 4,431–4,454)

*Xma*I *Nhe*I

SD-32 5'-GAG-CCCGGG-GCTAGC-CGGGACTAGGAGATGGGAAC-3' (nt 10,159–10,135)



SIV _{SMM-PBJ-4.41}	gag	pol	vif	vpx	vpr	tat	rev	su	tm	nef
HIV-1 _{BRU}	56	57	30	-	53	37	33	25	41	42
HIV-2 _{ROD}	86	82	73	81	71	63	64	69	74	60
SIV _{SMM-H-4}	94	98	92	92	94	89	91	86	92	81
SIV _{MAC-BK28}	91	92	82	90	89	77	75	83	79	70
SIV _{AGM-TYO-1}	58	57	36	35	-	37	48	46	45	44
SIV _{MND-GB1}	54	57	37	-	32	37	28	30	41	40

Immediately after amplification, SDS and EDTA were added to the PCR reaction (to 0.1% and 5 mM, respectively), and DNA was precipitated with 70% ethanol and 2.5 M NH₃OAc. After restriction enzyme digestion with *Nhe*I and *Hind*III and electrophoresis, desired fragments were gel-isolated using glass beads (BIO101), ligated to predigested BlueScript (StrataGene) plasmid and transformed into *Escherichia coli* strain JM109 by electroporation. Plasmids were then subcloned into the dam⁻ *E. coli* strain JM110 to allow regeneration of full-length virus using the methylation-sensitive restriction enzyme *Bcl*I. To reduce loss of SIV inserts, bacteria were grown at room temperature. After DEAE-mediated transfection of plasmid DNA into CEMx174 cells²⁰, virus was detected by reverse transcriptase assays as described²¹.

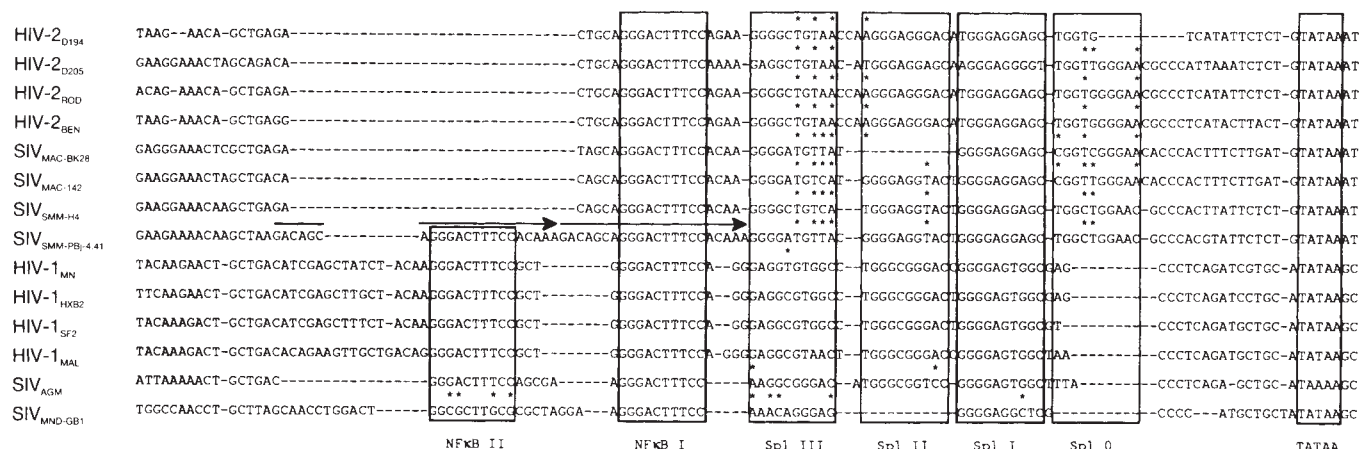


FIG. 2 Alignment of sequences within the upstream enhancer region of divergent HIV-1, HIV-2 and SIV LTRs. Gaps are indicated by dashes and are introduced to maintain alignment of similar sequences and to delineate regions corresponding to transcription factor binding site consensus sequences. Arrows indicate the position of the 22-bp direct repeat in SIV_{SMM-PBJ-4.41}. Regions corresponding to transcription factors Sp1 ((G/T)RGG(C/A)G-

(G/T)RRY; R, unspecified purine; Y, unspecified pyrimidine) and NF-κB (GGGACTTTCC) binding sites and to the promoter (TATAA) are boxed. Bases which do not match the consensus are highlighted with an asterisk. Data were taken from the human retrovirus and AIDS database at the Los Alamos National Laboratory.

examined had a prematurely truncated transmembrane protein caused by insertion of a stop codon, despite passage in human cells, which often selects for this change^{4,5,9}. The *nef* gene is truncated in SIV_{SMM-PBJ} clones relative to that in SIV_{SMM} clones H3 and H4, although the putative GTP-binding domain is well-conserved.

The most divergent region between SIV_{SMM-PBJ-4.41} and other SIVs lies in the U3 region of the LTR, where prototypical clones differ principally by the number of copies of sequences thought to be involved in transcription-factor binding. All three SIV_{SMM-PBJ} 3' clones examined had a 22-bp duplication in the enhancer region, which includes a possible NF-κB binding site in addition to the single NF-κB site found in most reported SIV and human immunodeficiency virus-2 (HIV-2) isolates, and similar to that found in all reported HIV-1 isolates (Fig. 2).

Molecularly cloned SIV isolates so far analysed have been minimally pathogenic^{10,11} (M. Murphey-Corb *et al.*, unpublished results). To determine whether the molecularly cloned SIV_{SMM-PBJ} viruses induced some or all aspects of the acute disease caused by the original strain, two adult pig-tailed

macaques were each inoculated intravenously with 1 ml virus stocks derived from the molecular clones. All three cloned viruses were pathogenic; they induced bloody mucoid diarrhoea in all animals within 3 to 7 days post-inoculation, a hallmark of SIV_{SMM-PBJ} disease (Table 1). Only three animals, two of which received the SIV_{SMM-PBJ-4.41} clone, died rapidly. Two additional animals died on day 49 and day 55 post-inoculation; both had diarrhoea, severe progressive weight loss and marked lymphopenia with decreased numbers of total T cells as well as CD4⁺ and CD8⁺ T cells. At death, one of these animals had severe degenerative myocarditis and the other had oroesophageal candidiasis. Although the course of disease after inoculation with SIV_{SMM-PBJ} or its molecular clones was accelerated compared with that induced by other SIV isolates described so far, these animals developed symptoms common to human or simian AIDS, including loss of significant numbers of T and B lymphocytes (Fig. 3a). Animals that survived longer, including two rhesus macaques (see below), had similar decreases in absolute numbers of T and B lymphocytes within the first week after inoculation, but to a lesser extent (data not shown).

TABLE 2 Virus status and antibody titres in recipients of SIV_{SMM-PBJ} clones

Animal	Plasma viraemia		PBMC	Virus isolation†				Serum antibodies	
	TCID ₅₀ ml ⁻¹	PI*		MLN	Spl	CSF	Cbr	Titre	PI*
90E	ND‡	ND	ND	+	+	+	+	ND	
F165	5 × 10 ⁴	6 days	+	+	+	+	+	ND	
T323	5 × 10 ²	8 days	+	+	ND	+	+	ND	
F124	<2	9 days	+	ND	ND	ND	ND	1,600	55 days
T325	<2	9 days	+	+	+	ND	-	12,800	46 days
7D	5	9 days	+					12,800	18 weeks
RTp	<2	7 days	+					6,400	12 weeks
RMp	<2	7 days	+					<100	12 weeks

Plasma viraemia titres were determined by infection of 5 × 10⁶ human PBMC in duplicate wells of 12-well plates with 0.2 ml undiluted or 10-fold serial dilutions of plasma from each animal. Plasma was cryopreserved until use, and titrations for all animals were performed on the same day with PBMC from the same human donor. Cultures, to which fresh PBMC were added periodically, were monitored for 37 days for the presence of virus by reverse transcriptase assay. Antibody titres were determined with an HIV-2 EIA Kit (Genetic Systems) which has a high degree of cross-reactivity to SIV.

* Time post-inoculation (PI) in days or weeks.

† Virus was isolated at death or at other times PI from PBMC or tissues by coculture with 1 × 10⁷ normal human PBMC (stimulated for 3 days with PHA) in RPMI 1640 medium with supplements. Tissues (MLN, mesenteric lymph node; Spl, spleen; CSF, cerebral spinal fluid; Cbr, cerebrum) were minced with scissors and tissue fragments were cocultivated with normal human PBMC. All cultures were monitored for the presence of virus by RT activity: +, culture virus positive; -, culture maintained for 6 weeks before being discarded as negative. Macaque 90E died on day 6 PI before blood could be collected for analysis.

‡ ND, not determined.

Previous studies¹ (P. B. Jahrling, personal communication) showed that rhesus macaques are less susceptible than pig-tailed macaques to the pathogenic effects of SIV_{SMM-PBj}. Similarly, when two rhesus macaques were inoculated with the molecularly cloned SIV_{SMM-PBj-4.41} virus, neither animal developed diarrhoea or any evidence of disease over the next 5 months at least (Table 1). The mechanism of this apparent resistance is not known, but it is possible that replication of SIV_{SMM-PBj} is more restricted in rhesus compared with pig-tailed macaques. At early times after inoculation, plasma was tested for cell-free virus; higher levels of viraemia corresponded to rapid death (Table 2). Virus was isolated from PBMC of rhesus macaque RMP only once (at 1 week post-inoculation) and, in contrast to all other animals that survived more than 8 days, this animal has not so far developed detectable antibodies to SIV, consistent with the

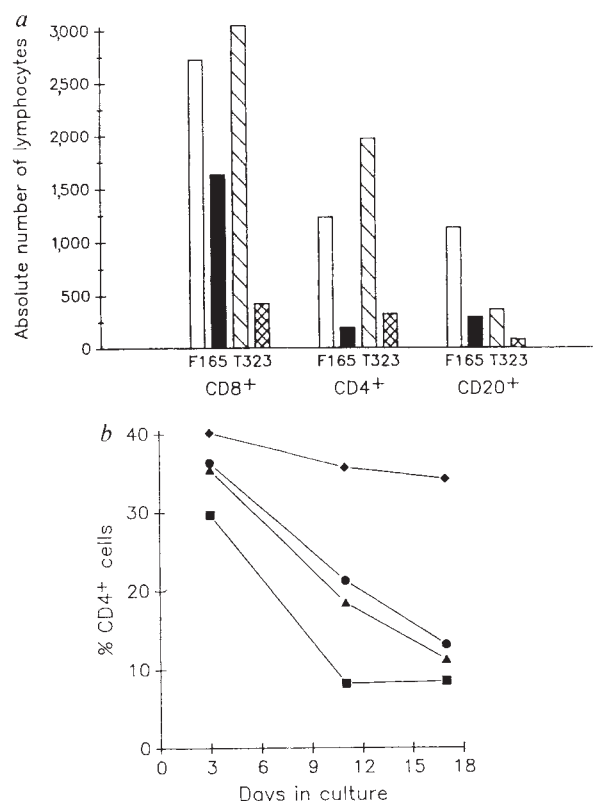


FIG. 3 *In vivo* and *in vitro* lymphocytopenia induced by SIV_{SMM-PBj} and its molecular clones. *a*, Loss of CD4⁺ and CD8⁺ T lymphocytes and CD20⁺ B lymphocytes from peripheral blood. Pig-tailed macaque F165 received SIV_{SMM-PBj-4.41} and died on day 6 PI; macaque T323 received SIV_{SMM-PBj-1.9} and died on day 8 PI. Absolute numbers of lymphocytes were determined by FACS analysis of whole blood using monoclonal antibodies Leu2a, Leu3a and Leu16 (Becton-Dickinson). Blood samples were obtained immediately before virus inoculation (□, ▨) and at the time the animals were killed (■, ▩) because of their moribund condition. *b*, Cytopathic effects on CD4⁺ cells from a sooty mangabey monkey. Shown are percentages of CD4⁺ cells in parallel cultures at different times after infection of PBMC from an SIV-seronegative, virus-negative mangabey with SIV_{SMM-9} (◆), SIV_{SMM-PBj} (■), SIV_{SMM-PBj-1.9} (●), and SIV_{SMM-PBj-4.41} (▲). After separation of heparinized blood on lymphocyte separation medium, PBMC were washed, and cultured in RPMI 1640 medium containing 10% fetal bovine serum, glutamine and antibiotics (10% RPMI). With no mitogen stimulation and no addition of interleukin-2 to the medium, 1.4×10^7 resting PBMC, in a volume of 2 ml medium, were infected with $\sim 200,000$ c.p.m. viral reverse transcriptase activity. At various times after infection, samples of culture medium were removed for determinations of reverse transcriptase activity and of total cell number, per cent viability and per cent CD4⁺ cells, by trypan blue dye exclusion and immunofluorescence assay, respectively. Fluorescein isothiocyanate-labelled Leu2a and phycoerythrin-labelled Leu3a were used to stain PBMC directly, and percentages of the two cell populations were determined by counting at least 500 cells with a Zeiss fluorescence microscope.

hypothesis that some rhesus macaques have an innate resistance to the acute effects of SIV_{SMM-PBj}.

At necropsy, histopathological findings in tissues from the three animals that died within 8 days were similar to those observed in animals that died rapidly after inoculation of the uncloned SIV_{SMM-PBj} pool, including hyperplasia of gut-associated lymphoid tissues¹. Virus was isolated from all tissues obtained at necropsy, including PBMC, spleen, mesenteric lymph node, cerebral spinal fluid and cerebrum (Table 2). In addition, viral DNA was demonstrated by PCR in small intestine, colon, spleen and mesenteric lymph nodes obtained from the three animals that died within 8 days (data not shown). Viral DNA was sufficiently abundant in gut-associated tissues to permit detection by Southern blotting (data not shown), indicating that molecularly cloned derivatives of SIV_{SMM-PBj} are enterotropic, consistent with previous studies of a molecularly cloned enterocytotropic and immunodeficiency-inducing strain of feline leukaemia virus¹².

SIV_{SMM-PBj} differs biologically from its parent virus, SIV_{SMM-9} (refs 1, 13) in several ways: SIV_{SMM-PBj} has an expanded host range, is not neutralized by serum from macaque PBj (the animal from which SIV_{SMM-PBj14} was isolated, 14 months after inoculation of SIV_{SMM-9}), and can form syncytia with Sup-T1 cells¹. Most important, prototypic SIV_{SMM} isolates from sooty mangabeys are not cytopathic for mangabey CD4⁺ cells *in vitro* (P.N.F., unpublished data). In contrast to SIV_{SMM-9}, its parent virus, SIV_{SMM-PBj} and its derivatives were uniquely cytopathic for mangabey CD4⁺ cells (Fig. 3*b*), suggesting that this property might be an *in vitro* correlate for induction of acute disease *in vivo*.

Our data show that acute disease and death can be induced by a lentivirus; that is, contrary to the implication of their name, lentiviruses are not limited to inducing slowly progressive diseases. Sequence analysis indicates that SIV_{SMM-PBj} acquired its acutely lethal phenotype through the accumulation of subtle mutational changes. Therefore, the possibility that lethal HIV variants exist with properties similar to those of SIV_{SMM-PBj} must be considered. It is conceivable that the spread and subsequent acute lethality of such a variant could be suppressed by previous infection with less acute variants. This hypothesis is supported by the finding that previous infection of pig-tailed macaques with SIV_{SMM-9} protects against acute disease on subsequent inoculation of SIV_{SMM-PBj} (P.N.F., D.C.A., H. M. McClure, unpublished data). In addition, the data suggest that two correlations may be made with the acute pathogenicity of SIV_{SMM-PBj}: *in vitro* cytopathicity for mangabey CD4⁺ cells and reiteration of transcription factor binding sites in SIV LTRs. As noted above, SIV_{SMM-PBj} has an additional NF- κ B binding site, and the minimally pathogenic BK28 isolate has lost a potentially critical Sp1 site (Fig. 2). Duplication of enhancer elements is associated with increased pathogenicity of murine and feline leukaemia retroviruses¹⁴⁻¹⁸; it is therefore possible that this duplication contributes to the acute course of disease induced by SIV_{SMM-PBj}. □

Received 22 February 1989; accepted 11 May 1990.

- Fultz, P. N., McClure, H. M., Anderson, D. C. & Switzer, W. M. *AIDS Res. Hum. Retroviruses* **5**, 397-409 (1989).
- Hirsch, V. M., Olmsted, R. A., Murphy-Corb, M., Purcell, R. H. & Johnson, P. R. *Nature* **339**, 389-392 (1989).
- Fukasawa, M. *et al. Nature* **333**, 457-461 (1988).
- Hirsch, V. M. *et al. Nature* **341**, 573-574 (1989).
- Kodama, T. *et al. J. Virol.* **63**, 4709-4714 (1989).
- Meyerhans, A. *et al. Cell* **58**, 901-910 (1989).
- Lasky, L. A. *et al. Cell* **50**, 975-985 (1987).
- Bosch, M. *et al. Science* **244**, 694-697 (1989).
- Chakrabarti, L., Emerman, M., Tiollais, P. & Sonigo, P. *J. Virol.* **63**, 4395-4403 (1989).
- Naidu, Y. M. *et al. J. Virol.* **62**, 4691-4696 (1988).
- Baier, M. *et al. J. Virol.* **63**, 5119-5123 (1989).
- Overbaugh, J., Donahue, P. R., Hoover, E. A., Quackenbush, S. L. & Mullins, J. I. *Science* **239**, 906-910 (1988).
- Fultz, P. N. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 5286-5290 (1986).
- Clark, S. P., Kaufhold, R., Chan, A. & Mak, T. W. *Virology* **144**, 481-494 (1985).
- Li, Y., Golemis, E., Hartley, J. W. & Hopkins, N. *J. Virol.* **61**, 693-700 (1987).

16. Chattopadhyay, S. K. *et al.* *Virology* **168**, 90–100 (1989).
17. Holland, C. A., Thomas, C. Y., Chattopadhyay, S. K., Koehne, C. & O'Donnell, P. V. *J. Virol.* **63**, 1284–1292 (1989).
18. Fulton, R., Plumb, M., Shield, L. & Neil, J. C. *J. Virol.* **64**, 1675–1682 (1990).
19. Brutlag, D. L., Dautricourt, J.-P., Maulik, S. & Relp, J. *Comput. appl. Biosci.* (in the press).
20. Dorsett, D. L., Ilana, K. & Winocour, E. *J. Virol.* **48**, 218–228 (1983).
21. Goff, S. P., Trackman, P. & Baltimore, D. *J. Virol.* **38**, 239–248 (1981).

ACKNOWLEDGEMENTS. We thank C. Horaist, T. Welch, M. Duda, B. Switzer and A. Brodie for technical assistance, B. Siegel for graphics, K. Kusumi and Drs A. deRonde and J. Sninsky for helpful discussions, Cetus Corporation for the gift of *Taq* polymerase and the use of the Thermal Cycler, and Dr P. Luciw for CEMx174 cells. The nucleotide sequences described here have been deposited in the GenBank/EMBL database under the accession number M31325. This work was supported by the NIH and the United States Army Medical Research and Development Command. S.D. is a scholar of the American Foundation for AIDS Research. J.E.E. is a recipient of an NRSA postdoctoral fellowship.

Activation of transcription by HIV-1 Tat protein tethered to nascent RNA through another protein

Christopher Southgate, Maria L. Zapp & Michael R. Green*

Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, Massachusetts 02138, USA

* To whom correspondence should be addressed

THE human immunodeficiency virus type I (HIV-1) nuclear protein Tat is a potent activator of viral gene transcription^{1,2}. Activation by Tat requires a *cis*-acting element, the transactivation response (TAR) site, located immediately downstream of the transcription start site^{3–5}. Several observations suggest that TAR functions as the nascent RNA product of the HIV long-terminal-repeat promoter (for a review, see ref. 6). Indeed, Tat protein and several cellular proteins bind directly to nascent TAR RNA *in vitro*^{7–10}. The significance of these *in vitro* interactions remains to be established. Here we report that Tat can activate transcription when bound to nascent RNA through the RNA-binding domain of another HIV-1 protein, Rev. Rev is a sequence-specific RNA-binding protein, which interacts with the viral RNA element RRE (refs 11–15). A Tat–Rev fusion protein efficiently activates transcription from an HIV-1 promoter derivative, in which TAR has been replaced by the RRE. We conclude that activation of tran-

scription by Tat can occur by direct binding to nascent RNA, and that the sole function of TAR may be to provide a Tat-binding site. Our results further suggest that cellular proteins that bind specifically to TAR RNA or TAR DNA may not be essential for Tat-responsiveness.

Protein-fusion strategies have been used to define functional domains of transcription factors, determine the role of their binding sites, and test models of transcription factor function (reviewed in ref. 16). To study the mechanism of Tat action we carried out the protein-fusion experiment of Fig. 1. The *tat* gene was fused in-frame to the HIV-1 *rev* gene so as to encode a Tat–Rev fusion protein. We then tested the ability of Tat–Rev to activate transcription from an HIV-1 promoter derivative in which TAR has been replaced by the RRE. As all TAR sequences have been deleted, cellular proteins that bind specifically to TAR RNA or TAR DNA should not interact with this promoter or its nascent RNA product.

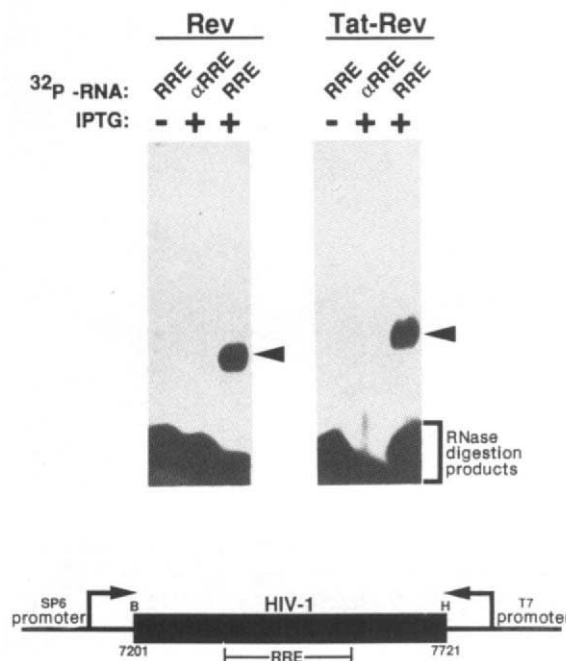
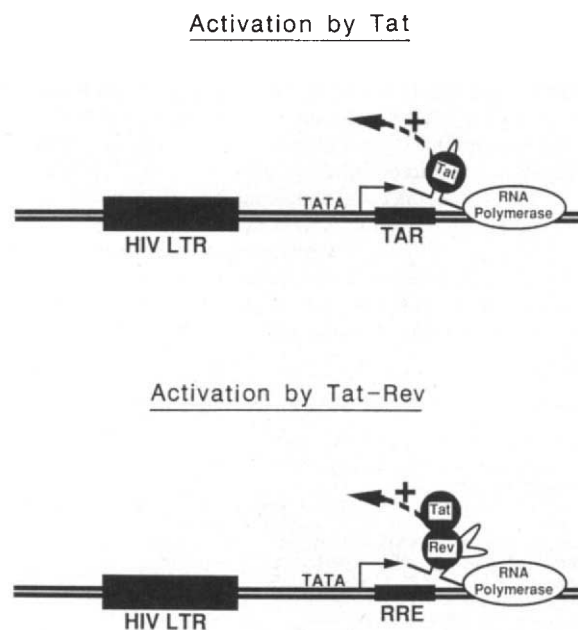


FIG. 2 An *E. coli*-derived Tat–Rev protein can specifically bind to the RRE *in vitro*. Extracts were prepared from noninduced (–) or isopropylthiogalactoside (IPTG)-treated (+) XA90 cells transformed with plasmid pZ76–Rev or pZ76 Tat–Rev. RNA-binding was analysed using an RNase protection/mobility shift assay as previously described¹¹. The ³²P-labelled RNA substrate used is indicated above each lane. RRE, sense RRE transcript; αRRE, antisense RRE transcript. Arrowheads, positions of the RNA–protein complexes. Sense and antisense RRE transcripts (³²P-labelled) were synthesized using SP6 RNA polymerase and T7 RNA polymerase, respectively, as indicated by the schematic diagram. Black box, HIV-1 sequences; B, *Bgl*II site; H, *Hind*III site. The minimal HIV-1 RRE (nucleotides 7,361–7,569) is indicated. METHODS. Plasmid pZ76 Tat–Rev, which expresses Tat–Rev in *E. coli*, was constructed by inserting HIV-1 sequences encoding Tat (amino acids 1–83) in-frame to the initiation codon of pRW76 (ref. 11). A DNA fragment containing HIV-1 sequences encoding Rev (amino acids 1–116) was then fused in-frame at the end of the *tat* sequence. Preparation of *E. coli* extracts and ³²P-labelled RNA substrates and details of the RNase protection/mobility shift assay were as previously described¹¹.

FIG. 1 Diagram of the experimental strategy. Top, model for transcriptional activation of the wild-type HIV-1 LTR by Tat. According to this model, Tat activates transcription after binding to nascent TAR RNA (reviewed in ref. 6; also see below). Bottom, activation, based on this model, by the Tat–Rev fusion protein after binding by means of the RNA-binding domain of Rev to the nascent RRE RNA.

To verify that the Tat-Rev fusion protein retained the sequence-specific RNA-binding activity of Rev, we performed an *in vitro* RNA-binding experiment. We expressed Tat-Rev in *Escherichia coli* and assayed its sequence-specific RNA-binding activity with an RRE-containing RNA substrate (Fig. 2) as previously described¹¹. The Tat-Rev fusion protein bound to RNA containing an RRE, but not to RNA containing antisense RRE sequences. When compared in parallel, the *E. coli*-derived Rev protein had the same binding specificity. We conclude that Tat-Rev can specifically bind to an RRE-containing RNA through the RNA-binding activity of Rev.

Figure 3 analyses the transcriptional activities of Tat, Rev and Tat-Rev after co-transfection with appropriate reporter plasmids into HeLa cells. The structures of the promoters and activator proteins are shown in Fig. 3a; the two reporters are the wild-type HIV-1 long terminal repeat (LTR) and the HIV-1 LTR-RRE. Transcription from each reporter was measured by an RNase protection assay in the presence and absence of an activator protein. Accurately initiated RNA from the wild-type HIV-1 LTR and the HIV-1 LTR-RRE gave rise to RNase-resistant RNA fragments of 84 and 226 nucleotides (nt), respectively. A co-transfected human α -globin gene was an internal control for transfection efficiency and RNA recovery.

As expected, Tat efficiently stimulates transcription from the wild-type HIV-1 LTR (Fig. 3b). Transcription from this LTR was stimulated to a comparable level by Tat-Rev, indicating that the fusion protein retains full Tat activity. Transcription from the HIV-1 LTR-RRE was not activated by Tat, as expected

from previous studies demonstrating that deletion of TAR eliminates Tat-inducibility. Despite the presence of the RRE, transcription of this reporter was not stimulated by Rev, a regulatory protein that acts post-transcriptionally (reviewed in ref. 17). The Tat-Rev fusion protein, however, significantly stimulated transcription from the HIV-1 LTR-RRE promoter. Neither Tat nor Tat-Rev stimulated transcription of the co-transfected human α -globin gene. We conclude that Tat-Rev was directed to nascent RRE RNA through the RNA-binding activity of Rev, enabling Tat to stimulate transcription.

For additional evidence that transcriptional activation by Tat-Rev involves sequence-specific RNA binding, we tested the ability of Tat-Rev to activate transcription from the reporter HIV-1 Δ TAR-CAT, which does not contain either a TAR or an RRE (Fig. 4, bottom panel). To detect and quantitate accurately the low level of transcription in the absence of an activator, we measured transcriptional activity by a chloramphenicol acetyltransferase (CAT) assay. Consistent with the results in Fig. 3b, both Tat and Tat-Rev activated transcription from the wild-type HIV-1 LTR. By contrast, neither Tat, Rev nor Tat-Rev could stimulate transcription from HIV-1 Δ TAR-CAT. Thus, activation by Tat-Rev requires that the reporter contains either TAR or the RRE.

On the basis of these results, we can draw several conclusions about the mechanism of Tat action. First, as the specificity of Tat has been redirected through an RNA-binding protein, we conclude that Tat can indeed function by binding to nascent RNA downstream of the transcription start site. Second, as there

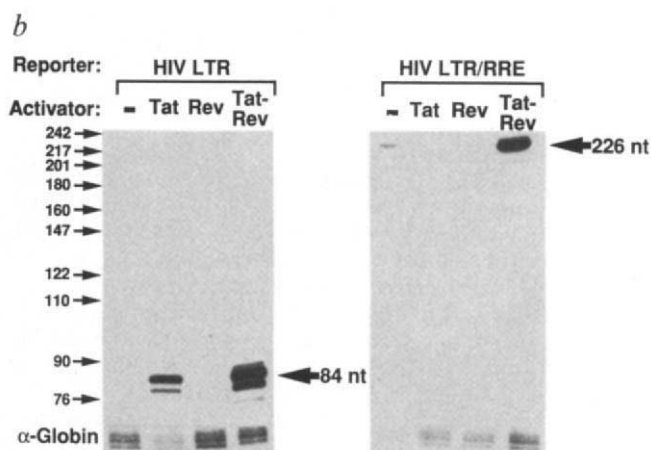
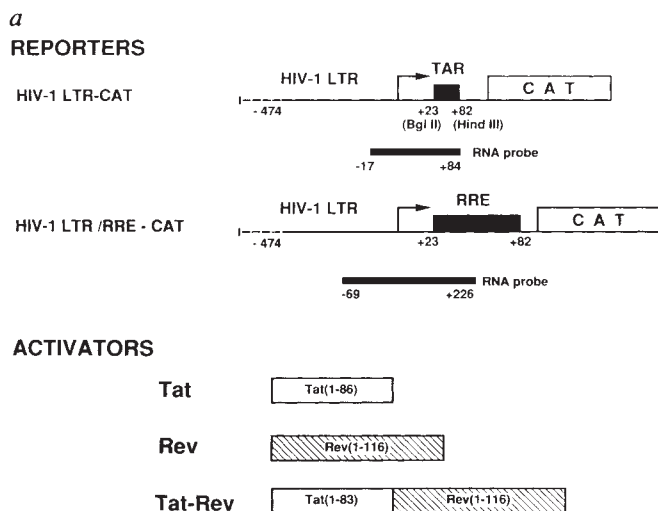
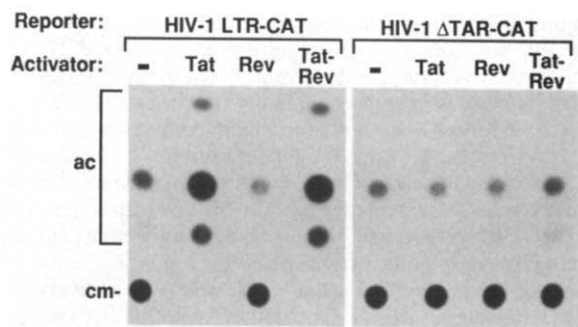


FIG. 3 Transcriptional activities of Tat, Rev and Tat-Rev. **a**, Schematic representation of the reporters and activators. The wild-type HIV-1 LTR-CAT construct contains sequences between -474 and +82 (relative to the cap site) fused to the bacterial CAT gene. HIV-1 LTR/RRE-CAT was constructed by deleting the TAR element between the *Bgl*III and *Hind*III sites at nucleotides +23 and +82 and inserting a 517-bp *Mbo*II-*Hind*III DNA fragment containing the HIV-1 RRE. The ³²P-labelled antisense RNA probes used to map correctly initiated HIV-1 LTR transcripts are depicted by solid bars below each construct. Tat, Rev and Tat-Rev were expressed from the cytomegalovirus (CMV) promoter; Tat was expressed from plasmid pcTat (ref. 28) and Rev was expressed from pcRev (ref. 29). A plasmid directing the synthesis of the Tat-Rev fusion protein was constructed by cloning *tat* cDNA nucleotides 1-83 in-frame to the initiation codon of pcRev. **b**, RNase protection analysis. The reporter plasmids HIV-1 LTR-CAT and HIV-1 LTR/RRE-CAT were transfected into HeLa cells in the presence of either pSP72 CMV (-), pcTat, pcRev, or pcTat-Rev. The reporter and activator proteins used are indicated above the lanes. The positions of the 226-nucleotide (nt) RNase-resistant RNA fragment indicative of accurately initiated RNA from the HIV-1 LTR/RRE, and the 84-nt RNase-resistant RNA fragment indicative of accurately initiated RNA from the HIV-1 LTR are indicated on the right. Each transfection mixture

also contained a human α -globin internal reference gene (pSP6H α_1). RNase-resistant RNA fragments from accurately initiated human α -globin RNA are shown below. Migration of DNA size markers (in base pairs) is indicated on the left.

METHODS. A reporter plasmid (5 μ g), a plasmid directing the synthesis of an activator protein (5 μ g) and pSP6H α_1 (2 μ g) were transfected into HeLa cells using the calcium phosphate co-precipitation technique. After 16-24 h, the cells were washed and shocked for 3 min with 25% dimethylsulphoxide/Tris-buffered saline. The cells were washed extensively and incubated for a further 24-48 h. Cytoplasmic RNA was prepared as previously described and digested with RNase-free DNase before RNase-protection analysis. The respective gel-purified ³²P-labelled antisense RNA probes (**a**) were hybridized to 10 μ g of cytoplasmic RNA at 60 °C for 12-16 h. Then 350 μ l of 0.3 M NaCl, 10 mM Tris-HCl buffer, pH 7.5, 1 mM EDTA containing 6 μ g ml⁻¹ RNase A and 20 U ml⁻¹ RNase T1 were added and incubated for 60 min at 30 °C. After proteinase K digestion and phenol extraction RNase-resistant fragments were fractionated on a denaturing 8 M urea polyacrylamide gel and visualized by autoradiography. In a separate reaction, accurately initiated transcripts from the human α -globin gene were quantitated using a ³²P-labelled antisense α -globin RNA probe as previously described³⁰.



REPORTERS

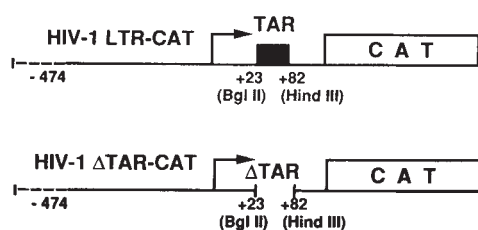


FIG. 4 Transcriptional activation by Tat-Rev is highly specific. Transfections into HeLa cells were as described in Fig. 3b. Cells were collected 48 h post-transfection and assayed for CAT activity. The reporter and activator proteins are indicated above each lane. Unacetylated and acetylated forms of [14 C]chloramphenicol were separated by thin-layer chromatography and detected by autoradiography. Schematic representations of the reporters are below. HIV-1 LTR-CAT is as described in Fig. 3a. The HIV-1 Δ TAR-CAT is identical to HIV-1 LTR-CAT, except that the sequence between positions +23 and +82, which includes the TAR element, has been deleted.

is no absolute requirement for the Tat-TAR interaction, we suggest that TAR is required only to direct Tat to the vicinity of the promoter. Thus, binding to TAR RNA does not, for example, convert Tat from an inactive to an active form. Third, cellular proteins reported to bind specifically to TAR RNA, and to TAR DNA, may be dispensable for Tat-responsiveness; these proteins should not bind to the RRE-containing promoter or its nascent RNA product. Such cellular proteins could assist Tat in binding to nascent TAR RNA. We note, however, that purified *E. coli*-derived Tat can by itself bind to TAR RNA *in vitro*⁷.

An alternative model for Tat function is that, rather than increasing the rate of transcription initiation, Tat overcomes premature termination at the TAR site^{18,19}. Evidence in support of this model includes the detection of short TAR-containing RNAs in the absence of Tat^{18,19}. In our experiments we have deleted TAR, the putative premature termination site, and maintained Tat-responsiveness.

During a typical virus infection or co-transfection experiment, it is likely that the HIV LTR undergoes several rounds of transcription. Nuclear 'run-on' experiments show that Tat increases the rate of transcription initiation (reviewed in ref. 6), but these experiments could not address which round(s) of transcription is stimulated. As Tat functions after binding to TAR RNA, the first round of transcription, which is required to produce the nascent TAR RNA, must be Tat-independent. Tat would then stimulate subsequent rounds of transcription, and thus acts on re-initiation rather than initiation.

Mutagenesis studies suggest that Tat contains several functional domains²⁰⁻²⁷. The protein-fusion approach described here provides a novel way of identifying the functional domains of Tat and define their functions. For example, mutations in the RNA-binding domain of Tat will eliminate the activity of Tat but not that of Tat-Rev; the RNA-binding domain of Rev will

be sufficient to direct Tat-Rev to the promoter. By contrast, mutations of the transcriptional effector domain of Tat will eliminate the activity of both Tat and Tat-Rev.

Our results also have implications for Rev function. Although strongly suggested by *in vitro* RNA-binding studies¹¹⁻¹⁵, our results demonstrate that Rev binds to the RRE *in vivo*. The assay for transcription activation by Tat-Rev will provide a complementary approach to characterize the functional domains of Rev. Similar protein-fusion experiments should be useful in studying other regulatory proteins that interact with RNA. □

Received 12 April; accepted 21 May 1990.

1. Arya, S. K., Guo, C., Josephs, S. J. & Wong-Staal, F. *Science* **229**, 69-73 (1985).
2. Sodroski, J. G., Rosen, C. A., Goh, W. C. & Haseltine, W. A. *Science* **228**, 1430-1434 (1985).
3. Rosen, C. A., Sodroski, J. G. & Haseltine, W. A. *Cell* **41**, 813-823 (1985).
4. Hauber, J. & Cullen, B. R. *J. Virol.* **62**, 673-679 (1988).
5. Jakobovits, A., Smith, D. H., Jakobovits, E. B. & Capon, D. J. *Molec. cell. Biol.* **8**, 2555-2561 (1988).
6. Sharp, P. A. & Marciniak, R. A. *Cell* **59**, 229-230 (1989).
7. Dingwall, C. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 6925-6929 (1989).
8. Gaynor, R., Soultanakis, E., Kuwabara, M., Garcia, J. & Sigman, D. S. *Proc. natn. Acad. Sci. U.S.A.* **86**, 4858-4862 (1989).
9. Gatignol, A., Kumar, A., Rabson, A. & Jeang, K.-T. *Proc. natn. Acad. Sci. U.S.A.* **86**, 7828-7832 (1989).
10. Marciniak, R. A., Garcia-Blanco, M. A. & Sharp, P. A. *Proc. natn. Acad. Sci. U.S.A.* **87**, 3624-3628 (1990).
11. Zapp, M. L. & Green, M. R. *Nature* **342**, 714-716 (1989).
12. Daly, T. J., Cook, K. S., Gary, G. S., Malone, T. E. & Rusche, J. R. *Nature* **342**, 816-819 (1989).
13. Cochrane, A. W., Chen, C.-H. & Rosen, C. A. *Proc. natn. Acad. Sci. U.S.A.* **87**, 1198-1202 (1990).
14. Malim, M. H. *et al. Cell* **60**, 675-683 (1990).
15. Heaphy, S. *et al. Cell* **60**, 785-793 (1990).
16. Ptashne, M. *Nature* **335**, 683-689 (1988).
17. Green, M. R. & Zapp, M. L. *Nature* **338**, 200-201 (1989).
18. Kao, S.-Y., Calman, A. F., Luciw, P. A. & Peterlin, B. M. *Nature* **330**, 489-493 (1987).
19. Selby, M. J., Bain, E. S., Luciw, P. A. & Peterlin, B. M. *Genes Dev.* **3**, 547-558 (1989).
20. Garcia, J. A., Harrich, D., Pearson, L., Mitsuyasu, R. & Gaynor, R. B. *EMBO J.* **7**, 3143-3147 (1988).
21. Sadaie, M. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 9224-9228 (1988).
22. Green, M. & Lowenstein, P. M. *Cell* **55**, 1179-1188 (1988).
23. Ruben, S. *et al. J. Virol.* **63**, 1-8 (1989).
24. Kuppuswamy, M., Subramanian, T., Srinivasan, A. & Chinnadurai, G. *Nucleic Acids Res.* **17**, 3551-3561 (1989).
25. Rappaport, J., Lee, S.-J., Khalili, K. & Wong-Staal, F. *New Biologist* **1**, 101-110 (1989).
26. Frankel, A. D., Biancalana, S. & Hudson, D. *Proc. natn. Acad. Sci. U.S.A.* **86**, 7397-7401 (1989).
27. Hauber, J., Malim, M. & Cullen, B. R. *J. Virol.* **63**, 1181-1187 (1989).
28. Cullen, B. R. *Cell* **46**, 973-982 (1986).
29. Malim, M. H., Hauber, J., Fenrick, R. & Cullen, B. R. *Nature* **335**, 181-183 (1988).
30. Charnay, P. *et al. Cell* **38**, 251-263 (1984).

ACKNOWLEDGEMENTS. We thank Drs B. Cullen and W. Greene for supplying HIV-1 plasmids. M.L.Z. was supported by an NIH postdoctoral fellowship. This work was supported by the NIH (M.R.G.).

Structure of the fibronectin type 1 module

Martin Baron*, David Norman*, Antony Willis† & Iain D. Campbell*

* Department of Biochemistry and † MRC Immunochimistry Unit, University of Oxford, South Parks Road, Oxford, OX1 3QU, UK

THE rapid accumulation of sequence data has provided insight into the evolution of proteins and led to the identification of 'mosaic proteins'^{1,2}. These proteins have evolved by duplication, insertion and deletion of a common pool of structural units or modules, yet their biological functions are diverse. They are involved in cell adhesion and migration³, embryogenesis⁴ and the pathways of blood clotting, fibrinolysis and complement^{5,6}. The modular units are defined by 'consensus sequences' which often include conserved disulphide bonds. Despite the available sequence information, little is known of the tertiary structure of mosaic proteins. If, however, the 'consensus structure' of the modules were known, valuable structural information could be inferred about a wide variety of proteins and biological systems. An important mosaic protein is fibronectin, an extracellular matrix protein that consists of three types of module (see refs 3, 7 for reviews). Here we describe the structure of the fibronectin type 1 module which appears twelve times in fibronectin and is also found in factor XII⁸ and tissue

plasminogen activator⁹. The module was produced using a yeast expression system and the structure was determined in solution using ¹H NMR. This methodology promises to be extremely powerful in the investigation of modules from a wide range of mosaic proteins.

The expression in yeast (*Saccharomyces cerevisiae*) and the purification of the seventh type 1 module of human fibronectin, has been described previously¹⁰. A phosphoglycerate kinase promoter^{11,12} and the α -factor leader sequence^{13,14} were used to direct the expressed protein into the culture medium. The expressed module corresponded to amino acids 431–478 (ref. 15) (referred to here as residues 1–48) and included all the type 1 consensus sequence and the linker connecting this module to the preceding type 2 repeat.

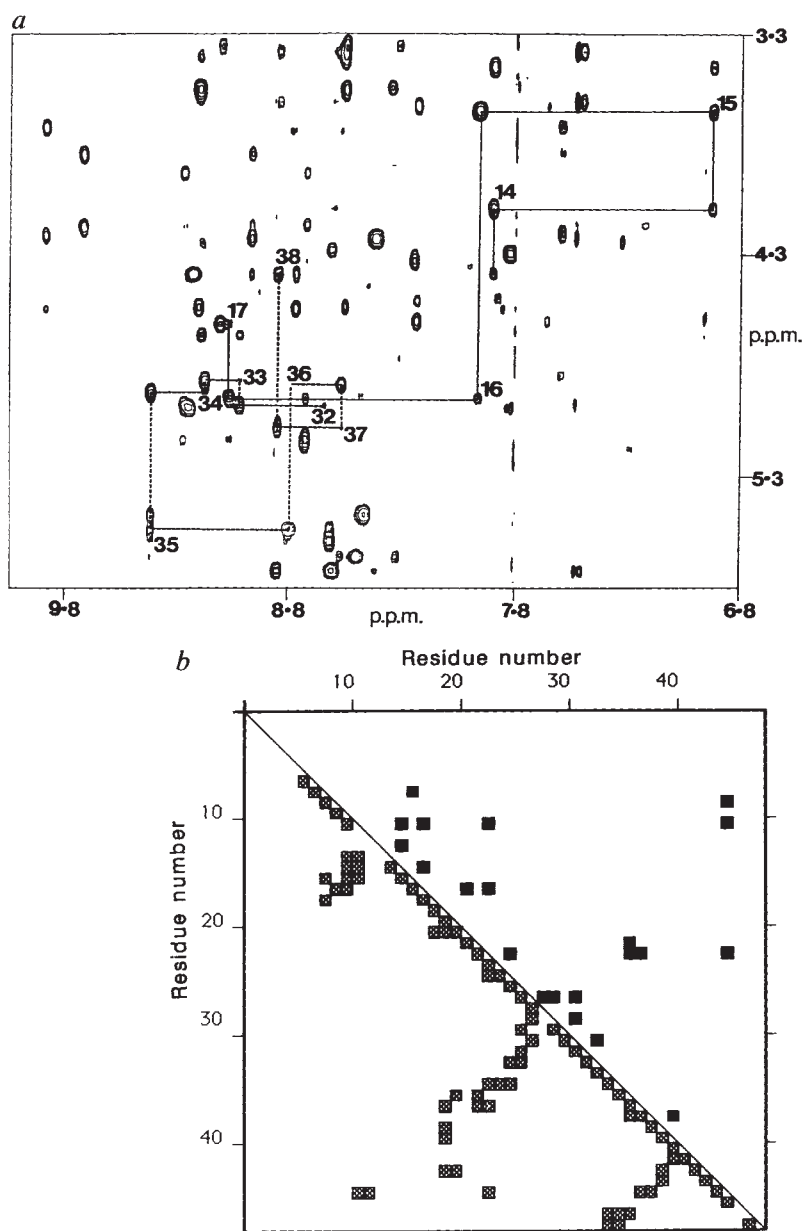
The methodology for assignment of protein NMR spectra and the determination of the structure of proteins in solution using ¹H NMR has been well described^{16,17}. In preliminary NMR investigations, we observed a substantial conformational change when the protein was titrated below pH 7.0, and it was therefore necessary to work close to a physiological pH. In spite of the non-optimum pH (lower pH is usually used to slow the exchange rate of amide protons with the solvent), 42 out of the 48 backbone amide protons were identified. Figure 1a is a region of a NOESY

(nuclear Overhauser enhancement spectroscopy) spectrum recorded in water at pH 7.6 showing nuclear Overhauser effects (NOEs) between the backbone amides and the alpha protons (through-space connectivities); 144 NOEs were short range ($< i, i+4$), allowing virtually complete sequence specific assignment of the protein (two sections of sequential assignment are highlighted in Fig. 1a). A further 104 NOEs were long range ($> i, i+4$), and the distribution of both long and short range NOEs is shown in Fig. 1b.

To calculate the three-dimensional structure, a combination of distance geometry followed by restrained molecular dynamics was used. The experimental input consisted of distance restraints derived from the observed NOEs. Because of relatively rapid amide proton exchange at pH 7.6, many of these resonances were broad and some were unobservable. Measurement of amide proton coupling constants to obtain torsion angle restraints and observation of slowly exchanging amide protons to identify hydrogen bonds were thus precluded.

Ten separate structures were calculated (Fig. 2a; Table 1); the final backbone structures are shown in Fig. 2a. Figure 3b is a schematic diagram of the secondary structure in which the type 1 consensus residues (determined by alignment of the human type 1 sequences in Fig. 3a) are highlighted.

FIG. 1 NMR data. *a*, A region of a NOESY spectrum showing NOEs from backbone amide protons. NMR spectra were collected on Bruker 600 MHz and 500 MHz spectrometers in both D₂O and H₂O at temperatures of 27 °C and 39 °C and pH 7.6 (uncorrected meter reading in D₂O). Two-dimensional NMR experiments were performed using standard Bruker microprograms; 64 scans were collected with 512 increments and 4,096 data points. NOESY spectra were recorded with mixing times of 100–300 ms. This spectrum was recorded with a mixing time of 300 ms, at a temperature of 27 °C; the protein concentration was 5 mM in 20 mM sodium phosphate buffer (pH 7.6). The solid and dotted lines illustrate the sequential assignment of two regions of the module (we refer to the residues in the expressed module as 1–48 and 430 must be added to obtain the numbering of intact fibronectin). Intra-residue $\text{HN}_i\text{-HC}_{\alpha i}$ NOEs are labelled. Vertical and horizontal lines connect these to unlabelled peaks which are sequential $\text{HN}_{(i+1)}\text{-HC}_{\alpha(i)}$ NOEs. *b*, Diagonal plot of the NOE distance constraints used for the generation and refinement of the fibronectin module 7 structures. The filled squares represent pairs of residues connected by at least one distance restraint. The solid squares indicate restraints involving only side-chain atoms and the hatched squares indicate restraints involving at least one backbone atom.



The dominant structural features of the module consist of two anti-parallel β -sheets, which is consistent with work on intact fibronectin using infrared spectroscopy¹⁸. Residues Ile 8–Thr 11 form a small double-stranded anti-parallel β -sheet with Val 15–Arg 18. The turn between Thr 11 and Val 15 is relatively poorly defined because the amide protons of Asn 12 and Glu 13 are not observed and so measurement of the dihedral angles was not possible. A loop search of the Brookhaven protein structure database found that the turn resembled a type I conformation. Following Arg 18, there is a short loop down into the first strand of the triple-stranded anti-parallel β -sheet and, within this loop, there is a conserved glycine residue (at position 20). The triple-stranded β -sheet is composed of strands

Asp 21–His 27, His 31–Cys 37 and Trp 45–Ile 48. The close proximity of His 27 and His 31 on the same surface of the β -sheet may explain the dependence of the structure on pH, as the conformational change occurs concomitantly with the titration of these histidines. The turn between His 27 and His 31 is well defined by NOEs between the side chains (the amide protons of Asp 28 and Met 29 were not observed). The turn was found to be of type I, with a highly conserved glycine (Gly 30) in a left-handed helical conformation¹⁹. The definition of the turn with respect to the remainder of the β -sheet is, however, less well defined. The large turn between the second and third strand of the main β -sheet is not highly restrained by experimentally observed NOE interactions, but seems to be a wide loop that

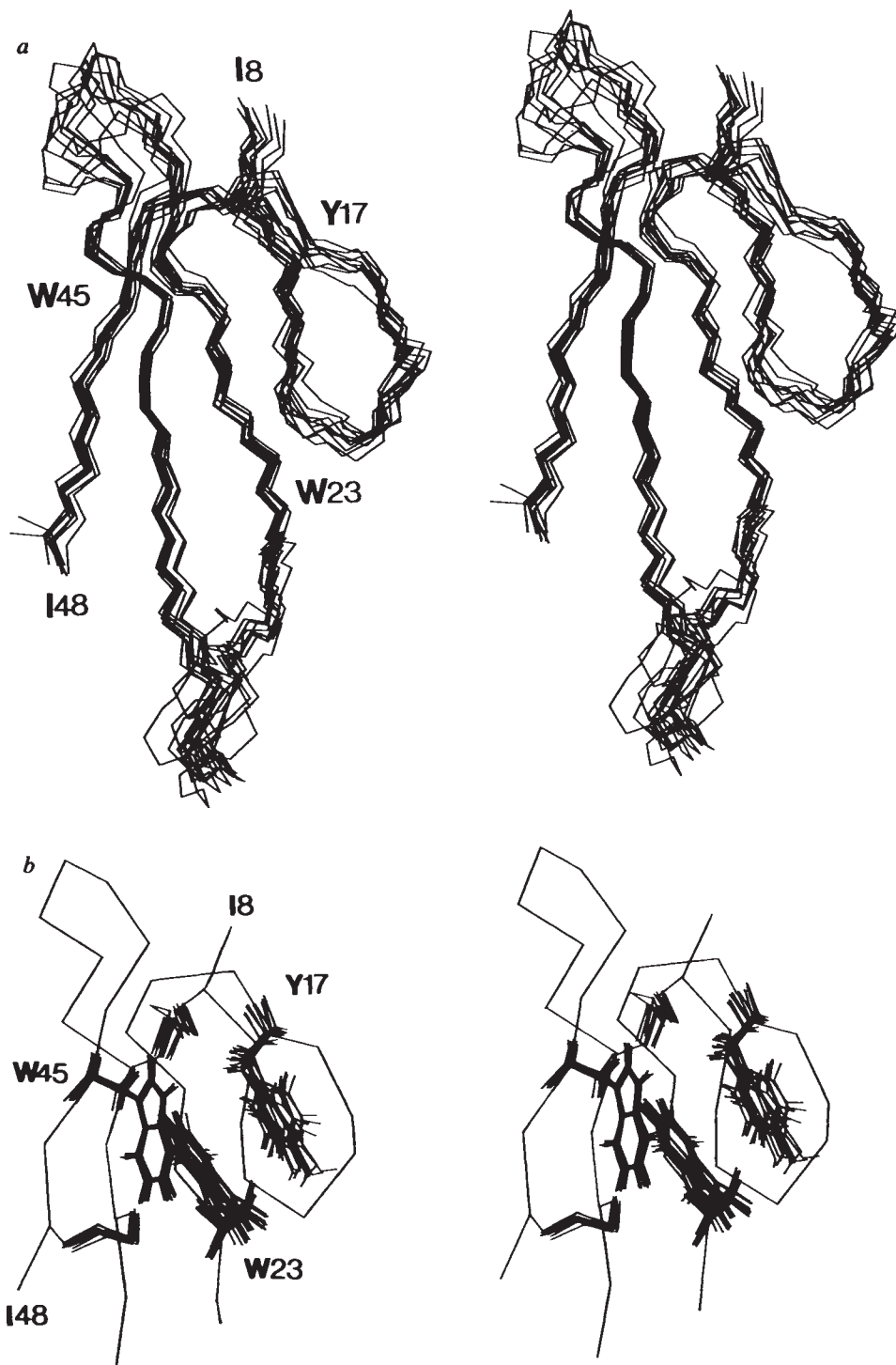


FIG. 2 Overlay structures. *a*, Stereo overlay of the C_{α} , C , N atom backbone (residues 8–48) of the ten final structures. Refinement was carried out with the inclusion of 381 experimentally determined distance restraints. The two disulphide bonds were also included. After preliminary calculations, 23 additional distance restraints were added to define hydrogen bonds predicted from the identified β -sheet secondary structure (those that were flanked by residues for which $HC_{\alpha}-HC_{\alpha}$ NOEs were observed, Fig. 3b). The hydrogen bond restraints were removed during the restrained molecular dynamics stage. NOEs were classified as strong (0.18–0.27 nm), medium (0.18–0.33 nm), and weak (0.18–0.5 nm) by measuring peak volumes in spectra recorded with mixing times of 100, 200 and 300 ms. Distances were calibrated to make the range of observed NOE intensities internally consistent. Initial structures were generated within the program DSPACE²¹ by distance geometry^{22–24}. Ten embedded structures were generated and then refined interactively²⁵ within DSPACE using a combination of conjugate gradient minimisation, coordinate randomization, and simulated annealing until the r.m.s. error due to the distance restraints was below 0.1 nm. The ten partially refined structures generated within DSPACE were transferred to full potential, restrained dynamics within the program XPLOR^{26–28}. The dielectric constant used within the coulombic potential was set to 80, and the explicit hydrogen bond potential restricted to backbone HN and O atoms only. The distance restraints were incorporated as square well, potentials with force constants of $1,740 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ for long-range and short-range restraints, and $420 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ for intraresidue restraints. The structures were first energy minimized for 400 cycles and then subjected to 2 ps of 27 °C restrained dynamics with a time step of 0.0005 ps followed by 20 ps of restrained molecular dynamics at 27 °C with a time step of 0.001 ps. The structures were finally energy minimized. *b*, An overlay of the core hydrophobic residues (Y17, W23, and W45) and the consensus disulphide bonds, with part of the average structure C_{α} backbone added to aid visualization.

shows considerable flexibility in restrained molecular dynamics simulations.

The β -sheets both have a right-handed twist and are stacked on top of one another, enclosing a hydrophobic core consisting of three highly conserved aromatic residues (Tyr 17, Trp 23 and Trp 45) and the two consensus disulphide bonds (Fig. 2b). The disulphide bridge between the first and third cysteines links the two β -sheet subdomains, and the disulphide bridge between the second and fourth cysteines links two adjacent strands of β -sheet.

Knowledge of this structure allows the prediction of those of the other type 1 modules and a more accurate alignment of the type 1 sequences than by sequence comparison alone. There are also implications for the way the modules may link together. The type 1 module occurs 12 times in fibronectin, in three clusters; six occur contiguously at the N terminus, three more follow two type 2 modules and a further three are found at the

C terminus. The module is rather elongated, with overall dimensions of approximately $1.7 \text{ nm} \times 1.6 \text{ nm} \times 3.2 \text{ nm}$. Dimensions of $\sim 2\text{--}2.5 \text{ nm} \times 120 \text{ nm}$ have been obtained by electron microscopy²⁰ for the intact fibronectin dimer. The dimensions of the single module are therefore consistent with an 'end-to-end' model for linking the type 1 structures together (although other microscopy studies¹⁸ have indicated a more globular structure). All the linker sequences between pairs of type 1 modules are short, apart from that between the fifth and sixth repeat. It is therefore interesting that as both the N- and C-terminal residues of each repeat lie in a β -sheet, there is the possibility that they could link together through a common β -strand. With five of the type 1 pairs (2-3, 3-4, 4-5, 8-9, and 10-11), if there were a common β -strand, it would pass directly from the C-terminal β -sheet into the N-terminal β -sheet of the following module. In the remaining type 1 pairs, the presence of additional residues in the linking sequences increases the

TABLE 1 Structural statistics

	Mean	Range
R.m.s. coordinate deviation from mean coordinate positions (nm)*		
Backbone atoms (C α , C, N, O)	0.0774	0.0762
All atoms (excluding hydrogens)	0.1318	0.0489
R.m.s. deviation from exptl restraints (nm)†		
All (381)	0.0254	0.0027
Long range (> $i, i+4$) (104)	0.0053	0.0015
Short range (< $i, i+4$) (144)	0.0100	0.0018
Intra residue (133)	0.0416	0.0043
R.m.s. deviations from idealized geometry‡		
Bonds (nm) (724)	0.0010	0.0001
Angles (deg) (1,292)	0.3060	0.0182
Impropers (deg) (241)	0.0349	0.0081
Potential energies (kJ mol ⁻¹)		
$F_{\text{TOT}}^{\S}$	611.15	130.84
F_{BOND}	38.69	10.81
F_{ANGLE}	745.75	88.40
F_{DIHEDRAL}	616.73	118.07
F_{IMPROPER}	18.47	8.35
F_{VDW}	-876.62	123.71
F_{ELEC}	-53.61	14.33
F_{HBOND}	-157.88	57.87
F_{NOE}	266.33	35.59

The statistics refer to the ten final structures (Fig. 2a, b) obtained in the manner described (Fig. 2a). Values in parentheses refer to the numbers of terms included.

* The r.m.s. coordinate deviation describes only residues 7-48 because of an almost total lack of restraints for the first seven residues.

† The r.m.s. deviation from experimental constraints refers effectively to deviations greater than the experimental distance restraints, as the chosen lower bounds are coincident with the sum of atomic volumes. Numbers in parentheses refer to the number of restraints in each category.

‡ The idealized geometry refers to that used within XPLOR. Figures in parentheses refer to the numbers in each category. Improper torsion potentials maintain chirality and planarity, including the *trans* peptide bond. The two disulphide bridges are included in the total potential as a combination of bond, angle, and improper terms.

§ The F values refer to the energies of the final structures, calculated using the CHARMM empirical energy functions. TOT is the sum of the partial potential energies present in the final structures; BOND, ANGLE, DIHEDRAL, and IMPROPER are energies describing the covalent geometry of the molecule. ELEC is the coulombic electrostatic energy calculated using a dielectric constant of 80; VDW is the Lennard-Jones, Van der Waals' energy; HBOND is the energy contribution from the explicit hydrogen-bond term; only the backbone HN and O atoms were included in this term; NOE is the energy due to violations of the upper bound limits of the experimental distance restraints. The potential used is the square-well potential with a force constant of $1,740 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ for long- and short-range distance restraints and $420 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ for the intra residue restraints.

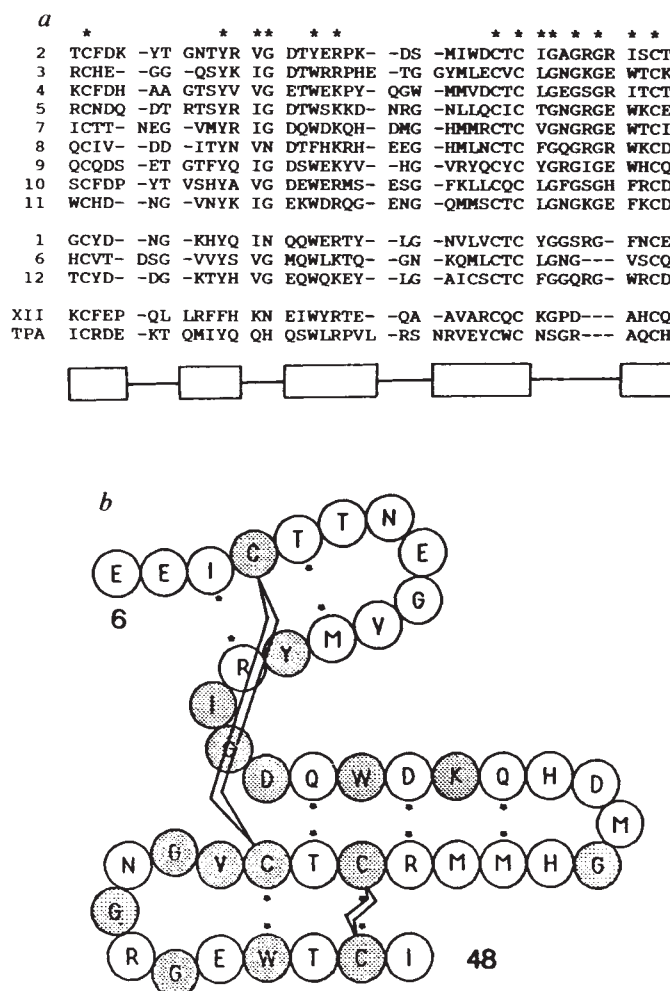


FIG. 3 Fibronectin structure. **a**, Alignment of human type 1 sequences from fibronectin (1-12), factor XII and tissue plasminogen activator (TPA). Using the determined structure (of module 7), those of the remaining type 1 modules can be predicted. The sequences are aligned to maintain homologous positions for highly conserved residues (marked with asterisks) in the secondary structure. Variations in distances between cysteines have been accommodated into the turns between the β -sheets. Spaces are inserted to divide the sequence into the secondary structure elements, and residues that are predicted to lie in β -sheets are indicated by boxes below the alignments. **b**, The secondary structure of the seventh type 1 module from human fibronectin. NOEs observed between C α protons are indicated by two asterisks. Shaded residues are highly conserved in aligned sequences or show predominantly conservative replacements.

range of conformations that may be modelled.

The type 1 module also occurs in factor XII and in tissue plasminogen activator. In the former it is sandwiched between two epidermal growth factor (EGF)-like modules and in the latter it occurs at the N terminus of the molecule adjacent to an EGF-like module. Like the type 1 module, the N- and C-terminal strands of EGF lie in β -sheets such that the type 1 module could also link into the EGF-like modules via a common β -strand.

Comparison of the type 1 sequences shows that those residues buried in the core of the structure are highly conserved, whereas those exposed to the solvent are both variable and predominantly hydrophilic, suggesting that these residues are also exposed to solvent in the intact molecule. One interesting exception is the type 1 module of tissue plasminogen activator. In this case, the lower surface of the principal β -sheet (the surface opposite to that which interacts with the minor sheet) has a large hydrophobic area consisting of Leu 22, Pro 24 and Leu 26 on the first strand, Val 31, Tyr 33 and Trp 35 on the second, and Val 46 and Val 48 on the third (numbering with respect to mature tissue plasminogen activator). This is in contrast to the opposing surface which has a number of hydrophilic residues (Arg 27, Arg 30, Glu 32, and Ser 45). It is possible, therefore, that the hydrophobic surface is shielded from the solvent in the intact molecule by interacting with one or more of the other modules of tissue plasminogen activator.

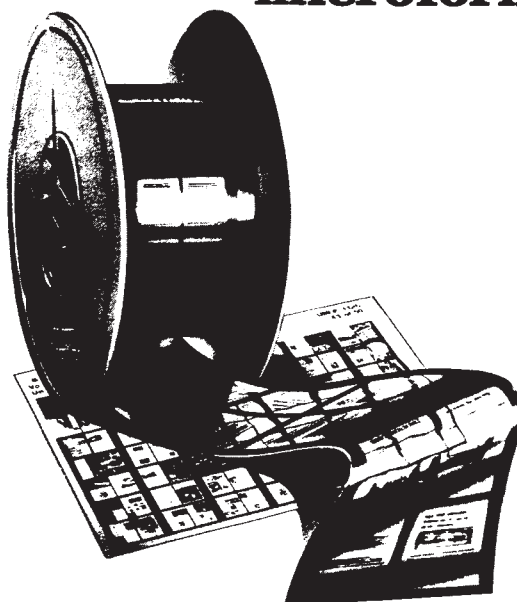
We are at present determining the structures of other types of module, which will allow models of many mosaic proteins to be constructed. For example, in addition to the type 1 module, the consensus structures are also known for all the other modules of tissue plasminogen activator. Although this immediately provides valuable information about local structure, the main problem in such modelling studies will be to determine the way the modules link together. One way of investigating these linkages would be to extend the methodology described here to the investigation of pairs of covalently linked modules. Models of mosaic proteins will aid the understanding of the structure/function relationships and the design and interpretation of module swapping and site-specific mutagenesis experiments. We are also using the expressed modules in biological assays and to raise antibodies to localize activities of the intact molecules. $\square$

Received 23 October 1989; accepted 22 March 1990.

1. Doolittle, R. F. *Trends Biochem. Sci.* **10**, 233-237 (1985).
2. Pathy, L. *Cell* **41**, 657-663 (1985).
3. Ruoslahti E. A. *Rev. Biochem.* **57**, 375-413 (1988).
4. Wharton, K. A., Johansen, K. M., Xu, T. & Artavanis-Tsakonas, S. *Cell* **43**, 567-581 (1985).
5. Furie, B. & Furie, B. C. *Cell* **53**, 505-518 (1988).
6. Reid, K. B. M. & Day, A. J. *Immun. Today* **10**, 177-180 (1989).
7. Hynes, R. O. & Yamada, K. M. *J. Cell Biol.* **95**, 369-377 (1982).
8. McMullen, B. A. & Fujikawa, K. *J. Biol. Chem.* **260**, 5328-5341 (1985).
9. Pennica, D. et al. *Nature* **301**, 214-221 (1983).
10. Baron, M., Kingsman, A. J., Kingsman, S. M. & Campbell, I. D. *Protein Production in Biotechnology* (ed. Harris, T. J. R.) 49-60 (Elsevier, London, 1990).
11. Mellor, J. et al. *Gene* **24**, 1-14 (1983).
12. Kingsman, A. J. & Kingsman, S. M. *Biotech. Genet. Eng. Rev.* **3**, 377-416 (1985).
13. Kurjan, J. & Herskowitz, I. *Cell* **30**, 933-943 (1982).
14. Brake, A. J. et al. *Proc. natn. Acad. Sci. U.S.A.* **81**, 4642-4646 (1984).
15. Owens, R. J. & Baralle, F. E. *EMBO J.* **5**, 2825-2830 (1986).
16. Wüthrich, K. *NMR of Proteins and Nucleic Acids* (Wiley, New York, 1986).
17. Clote, G. M. & Gronenborn, A. M. *Crit. Rev. biochem. molec. Biol.* **24**, 479-564 (1989).
18. Kotliansky, V. E. et al. *Eur. J. Biochem.* **119**, 619-624 (1981).
19. Sibanda, B. L., Blundell, T. L. & Thornton, J. M. *J. molec. Biol.* **206**, 759-777 (1989).
20. Odermat, E., Engel, J., Richter, H. & Hornmann, H. *J. molec. Biol.* **159**, 109-123 (1982).
21. Hare, D. R. & Morrison, R. D. *D-Space Manual* (Hare Research Inc., Woodville, USA, 1989).
22. Havel, T. F., Kuntz, I. D. & Crippen, G. M. *Bull. math. Biol.* **45**, 665-720 (1983).
23. Havel, T. F., Kuntz, I. D. & Crippen, G. M. *J. theor. Biol.* **104**, 383-400 (1983).
24. Crippen, G. M. *J. comput. Phys.* **24**, 96-104 (1977).
25. Weber, P. L., Morrison, R. & Hare, D. *J. molec. Biol.* **204**, 483-487 (1988).
26. Brünger, A. T. *X-PLOR manual* (Yale University Press, 1988).
27. Brünger, A. T., Kuryan, J. & Karplus, M. *Science* **235**, 458-460 (1987).
28. Brünger, A. T., Clote, G. M., Gronenborn, A. M. & Karplus, M. *Protein Eng.* **1**, 399-406 (1986).

ACKNOWLEDGEMENTS. We thank Tito Baralle for cDNA for human fibronectin, Sandra Fulton, Alan and Sue Kingsman for assistance with the molecular biology, and Rob Cooke, Nick Soffe and Jonathan Boyd for help with the NMR; Tony Fallon and Mark Edwards (British Biotechnology Ltd) for advice and oligonucleotides for vector constructions. This is a contribution from the Oxford Centre for Molecular Sciences which is supported by the SERC and the MRC.

nature is available in microform.



University Microfilms

International reproduces this publication in microform: microfiche and 16mm or 35mm film. For information about this publication or any of the more than 13,000 titles we offer, complete and mail the coupon to: University Microfilms International, 300 N. Zeeb Road, Ann Arbor, MI 48106. Call us toll-free for an immediate response: 800-521-3044. Or call collect in Michigan, Alaska and Hawaii: 313-761-4700.

☐ Please send information about these titles:

Name _____

Company/Institution _____

Address _____

City _____

State _____ Zip _____

Phone (____) _____

University
Microfilms
International